A Study of Physical, Chemical and Biological Properties of the Mushroom Casing Layer

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ABSTRACT

The function of the casing layer in the cultivation of the commercial white mushroom, Agaricus bisporus, was examined. The study was divided into 2 parts. Firstly, physical and chemical properties of 9 different casing materials were investigated and the findings related to the results of a cropping trial. Several materials, including granulated bark and mixtures of 'Fibre-mix' and granulated bark and 'Fibre-mix' and peat, proved to be suitable substitutes for the traditionally used peat casing. The structure of the casing material, especially the volume of air-filled pores, was found to be an important factor affecting the fruiting of A. bisporus. The volume of water held by a casing material bore no relation to yield. Secondly, biological aspects of the casing layer were examined. Bacteria, identified as Pseudomonas putida, were isolated from the casing layer and found to stimulate primordia formation and mycelial growth, but not strand development. Methods allowing the in vitro production of primordia were examined. A modification of Peerally's (1979) method which ensured low carbon-dioxide concentrations within the 'growth chambers' during sporophore initiation proved successful. Good evidence was found for the existence of a plasmid(s) in one of the bacterial isolates. The plasmid(s) appeared to be involved in the process of sporophore initiation and development in A. bisporus and also carried genes for mercury resistance.

ABBREVIATIONS

Ba	bark
%BS	percentage base saturation
CEC	cation exchange capacity
Fm	Fibre-mix
FmBa	Fibre-mix and bark (1:1)
FmPe	Fibre-mix and peat (1:1)
GBS	bark from Granulated Bark Supplies
KA	King's medium A
KB	King's medium B
LB	Luria-Bertani medium
Pe	peat
PeBa	peat and bark (1:1)
Pu	pumice
PuFm	pumice and Fibre-mix (1:1)
TEB	total exchangeable bases
TSI	triple sugar iron agar

GENERAL INTRODUCTION

A. bisporus, the most commonly grown mushroom commercially, is induced to fruit by application of a 3-5 cm layer of material such as buffered peat, soil, granulated bark to mycelium colonized compost (Long and Jacobs 1974). Failure to apply this 'casing layer' results in the development of few or no sporocarps (Pizer and Leaver 1947).

The casing layer is an extremely complex medium, its nature dependent on the interaction between a wide range of physical, chemical and biological factors. Despite considerable attention, the interaction between these factors and their effect on sporophore initiation and mushroom production is little understood. The major aim of the mushroom industry is to develop a system which enables cultivation of mushrooms in a more precise and controlled manner (Hayes and Nair 1976). The acheivement of this aim relies, to a large extent, on a greater understanding of the functioning of the casing layer.

This study is concerned with the elucidation of some of the physical, chemical and biological properties of the casing layer responsible for effecting the growth and fruiting of the cultivated mushroom, A. bisporus.

1.Ø CASING LAYER ASSESSMENT

1.1 INTRODUCTION

1.1.1 "EVOLUTION" OF THE CASING LAYER

The casing layer is an indispensible ingredient in the successful cultivation of A. bisporus. For more than 2ØØ years, mushroom growers have realized the need to cover the colonized compost with a layer of casing. The material most commonly used until recent times has been soil.

Experience taught early mushroom cultivators that the type of soil used as casing had a large effect on yield. Growers of this early period preferred either a steam sterilized loam top-soil, or a pest and disease free sub-soil (Edwards 1974). In both cases good structure was viewed as important. The benefits of using coarser textured soils was later shown by Pizer and Leaver (1947), whose work indicated that such soils produced greater yields than did fine textured loam soils.

During the 195Ø's, as a result of the work of Edwards and Flegg (1954) and Flegg (1954) , growers in many countries changed from using a casing of soil to using peat mixed with chalk-lime.

The situation today is similar to that of the 196Ø's when peat became universally recognized as the most productive casing material. Its exceptional structural and water holding properties, makes it the most favoured and

apparently suitable medium for casing mushroom beds (Yeo and Hayes 1979). In spite of the many benefits of peat, there are a few mushroom growers throughout the world who successfully case with alternative materials. In Switzerland, for example, one producer uses a mixture of soil, weathered spent compost and ground tuff. A number of French farmers use ground marl and several growers in the U.S.A. use a loam top-soil (Nair and Bradley 1981). In New Zealand, one grower uses a pumice soil and several others use granulated bark. In remote areas of the Himalayas, decomposed cow manure provides a satisfactory casing material (Hayes 1981).

In many of these situations, economic and/or practical considerations have meant peat has not been a worthwhile proposition. Growers who case with peat are increasingly having to contend with a number of problems particularily:

(1) Cost. Top quality Irish and German peat as used by most mushroom growers is becoming increasingly more expensive. In many situations the use of peat is no longer economically viable. In Australia for example, each grower in 1980 spent between (Aust.) $20,000 and $150,000 on imported peat depending on farm size (Nair and Bradley 1981).

(2) Future supply. World supplies of quality sphagnum peat, as preferred for mushroom cultivation, are not vast. Mushroom growers in a large number of countries rely on the peat reserves of Germany , Ireland or Finland (Vedder 1978). Recently Yeo and Hayes (1979) stated that the Irish peat reserves, which are the major source of peat for the

British mushroom industry, may be depleted in less than 30 years. These two problems in particular have prompted investigations in several countries aimed at the development, and/or discovery of a new casing material which is at least as productive as peat.

1.1.1a The New Zealand situation

Within the New Zealand mushroom industry there is a similar requirement for a study of alternative casing materials, however the need has arisen for different reasons.

Indigenous peat is the most commonly used casing material. New Zealand possesses two peat reserves, one in Southland and one in Hauraki. New Zealand growers, therefore, who use peat as a casing material do not face the same problems that affect growers in other countries who import European peat. Mushroom farmers in New Zealand are confronted instead with a problem that arises as a result of the young age of the New Zealand peat reserves. In contrast to the geologically old German or Irish peats, the New Zealand peats, because of their relative youth, form only shallow surface deposits. These surface deposits, like any top-soil provide niches for many organisms. Some, for example certain pseudomonads, appear to be beneficial to the growth and development of A. bisporus (Hayes et al. 1969). Others however, e.g. nematodes, phorids and some bacteria and fungi, are mushroom pests. These antagonistic organisms, unless eliminated, can cause serious disease outbreaks on mushroom farms. Steam sterilization of the peat is usually

employed to combat these pests, however complete eradication of disease causing organisms is usually not possible. Steam sterilization also has the adverse effect of reducing the numbers of beneficial micro-organisms, and consequently decreasing yield (Hayes 1974). Hayes, also reported that yield reductions were not apparent when peat/chalk casing materials were pasteurized with methyl-bromide, although it is now rarely used because of its high toxicity.

New Zealand peat is also an extremely variable material (pers. comm. F.R. Sanderson, DSIR, Lincoln). This tends to create management problems for growers. The random selection of harvest sites by commercial peat harvesters is the major cause of this variability (Bates 1974). The relative youth of New Zealand's peat reserves is also a contributing factor. Discovery of a uniform material would help solve some of the management problems that presently hinder the mushroom industry.

Some growers are already using other casing materials. Several use a granulated pine bark/chalk mix and one grower finds a pumice- soil/chalk mix adequate. A measure of how these materials compare with the standard peat/chalk casing material would be extremely useful to New Zealand mushroom growers.

An additional and somewhat inevitable problem, that of the rising price of peat in New Zealand, highlights further the need to examine alternative casing materials.

1.1.2 REQIUREMENTS FOR A PRODUCTIVE CASING MATERIAL

A greater understanding of the requirements of a productive casing layer are paramount to the development of alternative casing materials (Hayes 1981). These requirements, to a certain extent, have been out-lined by Atkins (1974) and Hayes (1979), however because of the difficulties involved in measuring factors which are relevent to productivity, the stated requirements are often vague and general. A statement such as; a productive casing material should have adequate structure, water holding capacity, texture and pH, is often encountered. In addition it is now widely accepted that biological factors, (especially bacteria), play an important role in a productive casing material (Eger 1961, Mathew 1961, Hayes et al. 1969, Park and Agnihotri 1969a, Hume and Hayes 1972, Hayes and Nair 1976, Visscher 1979, Peerally 1979, 1981). The involvement of bacteria in the growth and successful development of A. bisporus is discussed in chapter 2. It should be noted, that a productive casing material, will, because of its physical and chemical properties, provide favourable ecological conditions not only for the mushroom mycelium, but also for the necessary microflora.

1.1.2a Abiotic properties of the casing layer

In 1950, Bels-Koning examined properties of casing soils and concluded that moisture holding capacity is an important factor for a productive casing material. Edwards and Flegg (1954) examined physical properties of soils, in particular

moisture holding capacity, pore space and moisture tension. From a series of experiments using casing mixtures of peat and sand in various proportions, (in order to give a range of pore sizes), they concluded that more freely available water in the casing material, ie. a low moisture tension, was associated with a large number of mushrooms and earlier cropping. Work by Flegg and also Reeve et al. in 1959 further supported the earlier findings that a large water holding capacity coupled with a structure able to withstand repeated waterings without collapsing are primary requirements of a good casing material. In a recent examination of properties of casing materials, Hayes (1981) produced results which indicated that water holding capacity and pore space while being important factors, may not be the most critical factors determining productivity.

Hayes and Nair (1974), in examining the role of carbon-dioxide and oxygen in the casing layer, found that a casing material with open structure produced greater yields than did a compacted casing material. Hayes and Nair ascribed the larger yield to the fact that aeration of the casing layer was improved, a factor that several workers have shown is important in sporophore formation (Bels Koning 195Ø, Edwards and Flegg 1954, Tschierpe 1972). Visscher (1975) in a contrasting paper, reported evidence to suggest that the reverse was true. He found a compact casing material yielded better than an open structured casing subject to two conditions:

(1) That the casing material was well watered during vegetative growth.

(2) That the casing layer was ruffled just prior to fructification. Visscher argued that a compacted casing layer impeded gas exchange between the casing layer and the air in the mushroom house causing a build-up of carbon-dioxide in the casing and hence encouraging mycelial growth. Long and Jacobs (1968), San Antonio and Thomas (1972) and Nair et al. (1976) showed that high carbon-dioxide levels (above Ø.1%) promote vegetative mycelial growth. Ruffling the casing layer was done in order to restore a good diffusion rate in the top layer of the casing, thus lowering carbon dioxide levels to below Ø.1% to encourage reproductive growth.

The chemical nature of the casing layer and its effect on productivity has been difficult to assess. Pizer and Leaver (1947) found that pH, available potassium and phosphate appeared to have no influence on yield, except when the pH was below 5.4. Ganney and Richardson (1974) found enormous variability between the chemical properties of twelve different casing materials. This led them to question whether or not any importance could be placed on the chemical properties of the casing layer.

The effects of soluble salts on fruiting was investigated by Flegg (1959) who found the characteristic response to the addition of soluble salts was a reduction in the number of mushrooms but those that formed were, on average, heavier. Flegg (1961) reported that salt accumulation over time was a natural phenomenon occurring

even on untreated mushroom beds. In assessing the affects of these natural salt accumulations, he concluded that under normal conditions, the natural accumulations of soluble salts was unlikely to affect yield. Yeo and Hayes (1979) and Hayes (1981) showed that soluble salt levels increased over time. In contrast to Flegg (1961) however, they suggested the accumulation of soluble salts in the casing layer, (as indicated by the increased electrical conductivity), maybe associated with the declining yield during cropping. Laboratory experiments by Hayes (1972), which showed that some cations present in the casing layer inhibited primordia formation gave support to this theory. Hayes (1981), when re-examining the role of chalk-lime in the casing layer found that the addition of calcium-carbonate, as well as raising the pH, increasing buffering capacity and discouraging weed moulds, also significantly suppressed the accumulation of soluble salts.

1.1.3 ALTERNATIVE CASING MATERIALS AND TECHNIQUES

One of the first papers produced in response to the need for alternative casing materials and techniques was by MacCanna and Flanagan (1972). Their investigations were restricted to Irish peat based media mixed with various types of lime-stone. They reported little difference between treatments, indicating that a number of peat types and mixtures are suitable for casing mushroom beds. In the same set of experiments they also examined alternative casing

techniques. They studied the effect of incorporating small amounts of spawn run compost into the casing and also the effect of casing at normal casing time, as against casing at spawning. They found that casing at spawning time with 'spawned casing' resulted in flushes being picked two weeks earlier than usual. Barnard (1974) reported a similar spectacular result after casing with 'spawned casing'. In spite of these findings, 'spawned casing' has not been widely adopted, mainly because of the danger of rapid disease spread (Ganney and Stanley-Evans 1973, Barnard 1974).

Since MacCanna and Flanagan's work there have been many reports of trials using alternative casing materials. Stoller (1979) experimented with spent mushroom compost. This is now used by some growers in the United States. A major draw-back associated with this medium, however, is the amount of land needed on which to spread the spent compost which must be left for 2-3 years until well broken down and then steam sterilized before re-use (Vedder 1978).

Visscher (1982), reported on various materials used in Holland. Hygromull, (an urea-formaldehyde-resins-foam that can be milled to the wanted fineness), when mixed with peat and lime, was found to be slightly more productive than a customary peat/lime casing. Unfortunately hygromull has become too expensive and consequently has not been used commercially. A by-product from the extraction of sugar from sugar-beet, 'schuimaarde', when managed correctly and mixed with peat and lime produced good results. Visscher also

investigated spruce and pine bark, granulated rockwool, chopped bricks, beer spills, glass pearls etc., with varying levels of success.

One of the most favourable peat substiutes, PPMB, a by product of the paper milling industry has been used in England. A major advantage of this material lies in the fact that it can be milled to any fineness, thus allowing some control over its physical properties. Several workers have examined its physical, chemical and biological properties and have concluded that it is an excellent substitute for peat (Yeo and Hayes 1979, Cresswell and Hayes 1979, Hayes 1981).

Nair and Bradley (1981) examined alternative materials in Australia. Among those studied were defibrated pine wood and bark, spent casing, spent compost and soil, all of which yielded as well or better than German peat. By-products from the sugar refining industry, sawdust and an Australian peat were also examined giving a mixture of results.

1.1.4 STUDY AIMS

This study, in an attempt to find a substitute for peat, aims to assess the suitability of a range of products to function as casing materials. Included in this range are three materials currently used by growers in this country; peat, a pumice soil and pine bark chips. Also included is a promising new medium; a modified bark product from New Zealand Forest Products.

Assessment was carried out in two parts:

(1) A cropping experiment was set up to investigate the effect of each casing material on yield.

(2) A range of physical and chemical properties of each casing material were measured in an attempt to elucidate important factors influencing the growth and development of the cultivated mushroom.

1.2 MATERIALS AND METHODS

1.2.1 CASING MATERIALS

Nine different materials were investigated:

(1) Pine bark (Ba), from Oderings Nursery, Christchurch. The bark was granulated, (approx. 10mm maximum chip size) and had been left outside to weather for at least six months.

(2) Pine bark (GBS), kindly supplied by Granulated Bark Supplies, Auckland. The bark was a mix of number 1 and 2 fines, (approx. 10mm maximum chip size).

(3) Peat (Pe), from Southland, kindly supplied by Smith Soil Industries, Christchurch. The Southland, (Otanomomo), peat, consisted largely of wire rush roots, sedge and some sphagnum moss, plus a small fraction of silt and clay.

(4) Fibre-mix (Fm), kindly supplied by New Zealand Forest Products, Kinleith. Fibre-mix is a by-product of a process involving the extraction of poly-phenolic resins from the bark of Pinus radiata.

(5) Pumice soil (Pu), a yellow-brown pumice soil, from Rotorua. The sub-soil, (B), horizon was used which consisted of gravelly pumice with a large silt and clay fraction. Kindly supplied by Ted Williams, Rotorua.

(6) Pumice/Fibre-mix (PuFm), in a 1:1 mix.

(7) Fibre-mix/Bark (FmBa), in a 1:1 mix.

(8) Fibre-mix/peat (FmPe), in a 1:1 mix.

(9) Peat/bark (PeBa), in a 1:1 mix.

Oxford chalk-lime was incorporated into the casing materials in a ratio of 1:3, (chalk-lime : casing material). The physical and chemical properties of the casing materials were measured both with and without the added lime. Only casing materials with added lime were used in the cropping trial.

1.2.2 PHYSICAL AND CHEMICAL PROPERTIES

1.2.2a Experimental design

The time involved in measuring physical and chemical properties of nine different materials, with and without lime meant that replicating more than twice, in most cases was not practicable. If the results after two replications were different by more than 5% the experiment was repeated until results were consistent. The uniformity of the materials measured was such that in most cases duplication was sufficient. In situations where it was warranted, larger samples were used in order to further reduce variability.

1.2.2b Hydrogen ion concentration (pH)

A 5Øml beaker was half filled with an equal volume of fresh casing material and distilled water. The solution was stirred for 2-3 min then allowed to stand for 3Ø min before measuring the pH with a glass electrode (Allen 1974). Measurements of pH were taken every four days throughout the duration of the cropping trial.

1.2.2c Buffering capacity

The mixture of casing material and distilled water used above was retained and 1ml aliquots of Ø.1N HCl were added to it. The solution was stirred and left for 5 min before noting the pH.

1.2.2d Electrical conductivity

Following the method of Yeo and Hayes (1979), 4 g of fresh casing material was stirred into 4Øml of deionized water, shaken for 1 min and then placed in a water bath at 2Ø C for 1 h. Electrical conductivity was measured using a YSI model 33 S-C-T meter. Throughout the duration of the cropping cropping trial, electrical conductivity was measured every fourth day.

1.2.2e Cation exchange properties

(1) Cation exchange capacity (CEC). The most widely used and accepted technique for evaluating the CEC of soils is the ammonium-acetate method (Metson and Blakemore 1968, Allen 1974). Complications, however, can sometimes occur when calcareous materials are analyzed by this technique because the extractant, (NH_4COOCH_3), can dissolve the $CaCO_3$ in the sample. Various modifications have been made by a number of workers but there is still much discussion as to their effectiveness (Metson and Blakemore 1964, Chapman 1965, pers. comm. J.A. Adams, Soil Science Dept., Lincoln College). In this study the general ammonium-acetate procedure was used for both limed and unlimed casing materials.

In an attempt to gain a measure of the CEC as close to the *in vivo* situation as possible, the sample of casing material was not ground and sieved. To compensate for the possibility that the unground sample may not be entirely homogeneous the sample size was increased from 10g to 25g.

The general procedure is shown in Fig 1.1. A standard was run through the Kjeldahl apparatus to provide an estimate of background ammonium-N which was used as a control. Knowledge of the amount of ammonium-N allowed calculation of the milli-equivalents of ammonium ion adsorbed per 100g of oven-dry soil, ie., the CEC (Metson and Blakemore 1964, Chapman 1965, Allen 1974).

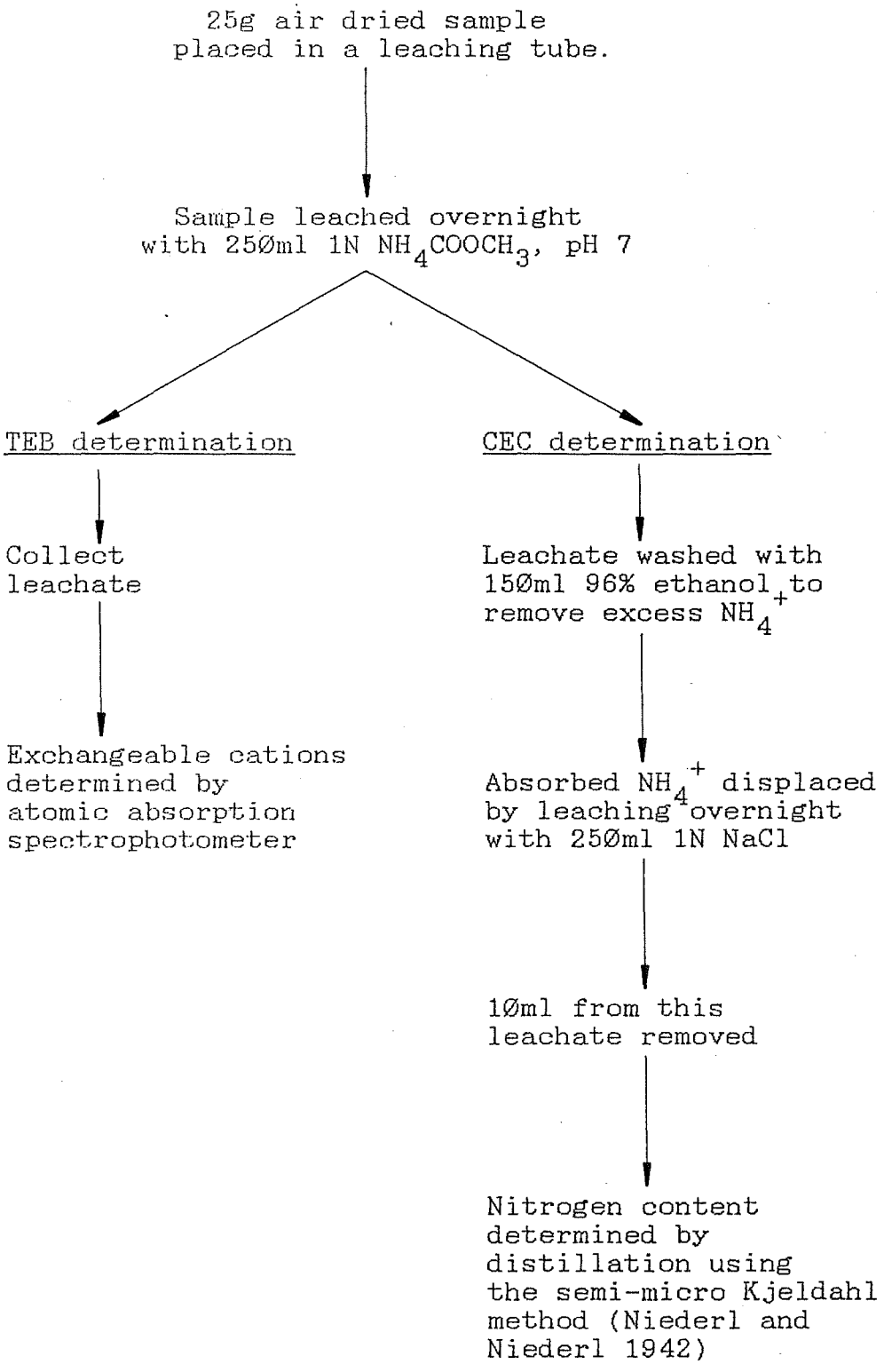
(2) Total exchangeable bases (TEB). The exchangeable cations were extracted and analysed using the procedure shown in Fig 1.1. The three major cations, Ca^{2+}, Mg^{2+} and K^{+}, were measured. Some minor cations were also investigated. Glass apparatus was used because of the lack of plastic equipment which meant a meaningful analysis of exchangeable Na^{+} was not possible. Summation of the individual cations gave an estimate of TEB (Allen 1974). The recommendation of Metson (1956) was followed for estimation of TEB in calcareous casing materials. TEB was assumed to be equal to CEC on the basis that any deficit of cations was made up by Ca^{2+} ions from the dissolution of $CaCO_3$

(3) Percentage base saturation (%BS). Calculation of %BS was made using the formula;

$$(TEB/CEC) * 100 = \%BS.$$

(Allen 1974).

Fig 1.1. Procedure for the determination of CEC and TEB



1.2.2f Moisture properties

A 2Øg sample, (approx.), of air dried casing material was weighed into a crucible. The sample was then placed in an oven at 1Ø5 C until a constant weight was reached. After removal from the oven the sample was placed in a desiccator to cool before weighing. Results from this enabled calculation of:

(1) The moisture factor. This allowed the analysis of physical and chemical properties to be carried out on air dry samples and the results converted to an oven-dry basis.

(2) The gravimetric water content; the percentage water content by weight expressed as a percentage of the oven-dry weight of material.

(3) The percentage dry matter in air dry soil. (Allen 1974). Refer to Appendix 1, (formulae) and Appendix 1a, (results).

1.2.2g Water holding properties

Water holding capacity was determined by weighing a dry glass tube, one end of which was covered with a filter paper held in place with a rubber band. The tube was filled to the top with casing material and reweighed. The full tube was then placed in a beaker containing water to within 1cm of the top of the tube and left until saturated. The tube was then removed, wiped dry and quickly weighed. These results allowed calculation of water holding capacity on a weight basis.

Expression of the water content on a volume basis was acheived using the bulk density of the casing as a means of

calculating the volume of a known mass of casing material, (Peters 1965).

An estimation of field capacity was acheived by determining the water content of samples of fresh casing material. As this involved taking samples from mushroom beds it was only possible to provide an estimate of the field capacity of the casing materials where lime had been added.

1.2.2h Bulk density

A known weight of air dry casing material was transferred in stages to a 50ml measuring cylinder, tapping down firmly between transfers. The volume was then recorded. Bulk density was calculated using the formula:

$$\text{Bulk density} = \frac{\text{mass of casing material (g)}}{\text{volume of casing material (cm}^3\text{)}}$$

1.2.2i Particle density

A clean dry 100ml volumetric flask was weighed, 50 g of casing material placed in it, and reweighed. Approximately 50 ml of water was then poured into the flask and the contents boiled gently for 2-3 min to remove any trapped air in the sample. After cooling to room temperature, cold boiled distilled water was added to the flask up to the 100 ml mark and then reweighed. Finally the contents of the flask were washed out and the flask filled with cold, boiled distilled water to the 100 ml mark and weighed.

The particle density was calculated by dividing the weight of casing material by the weight of water displaced by the casing material (Allen 1974, Blake 1965).

1.2.2j Porosity

The percentage total porosity was calculated after determination of bulk and particle density using the relationship:

$$\text{Porosity (\%)} = \frac{\text{Particle density - Bulk density x 100}}{\text{Particle density}}$$

(Allen 1974).

1.2.2k Air filled pores

The two formulae:

$$P_v = P_w \times D, \qquad S = S_t - P_v$$

where P_v = Water content on a volume basis

P_w = Water content on a weight basis

D = Bulk density

S = air-filled pore space

S_t = total porosity

allowed calculation of the air-filled pore space, see Vomocil (1965).

1.2.3 THE EFFECT OF THE CASING MATERIAL ON MYCELIAL MASS AND RATE OF MYCELIAL GROWTH

1.2.3a Rate of growth of mushroom mycelium through the casing

Glass tubes, (2Ø mm inside diameter), were plugged at one end with cotton wool. Five grams of mushroom spawn, (Ax6Ø), was placed on top of the cotton wool and then covered with casing material to a depth of 1ØØ mm. Triplicate tubes were set up and measurements were taken every 2-3 days.

1.2.3b Measurement of mycelial mass in the casing layer

This experiment was designed to provide an estimate of the mass of mycelial strands in the casing layer. It was not intended to provide a highly accurate measure of mycelial biomass and consequently the method outlined below should be viewed with respect to this aim.

A sample of casing material was removed from the mushroom beds at the end of cropping and weighed. Knowledge of the bulk density allowed calculation of the volume of casing material taken. The sample was placed in a container with 200 ml of 1% sodium lauryl sulphate in water and shaken vigorously for 2-3 min The container was then left for 20 min to allow the mycelial strands to float to the top. The strands were removed and blotted dry, any large clinging pieces of casing material were removed with forceps. In most cases, after a brief clean the mycelial strands still had a small amount of casing material attached. In order to compensate for this, correction factors were calculated as follows:

The percentage contribution of casing material to the fresh weight of the samples was calculated by careful cleaning of a representative sample. The percentage contribution of pumice to the fresh weight of the mycelium was determined by ashing. Four replicates of each casing material were used. The results were analysed using Statcalc (Lee et al. 1984).

1.2.4 CROPPING TRIAL

1.2.4a Experimental design and considerations

Mushroom cropping experiments often present many problems which, unless considered in the experimental design and procedure, can lead to invalid or misleading results.

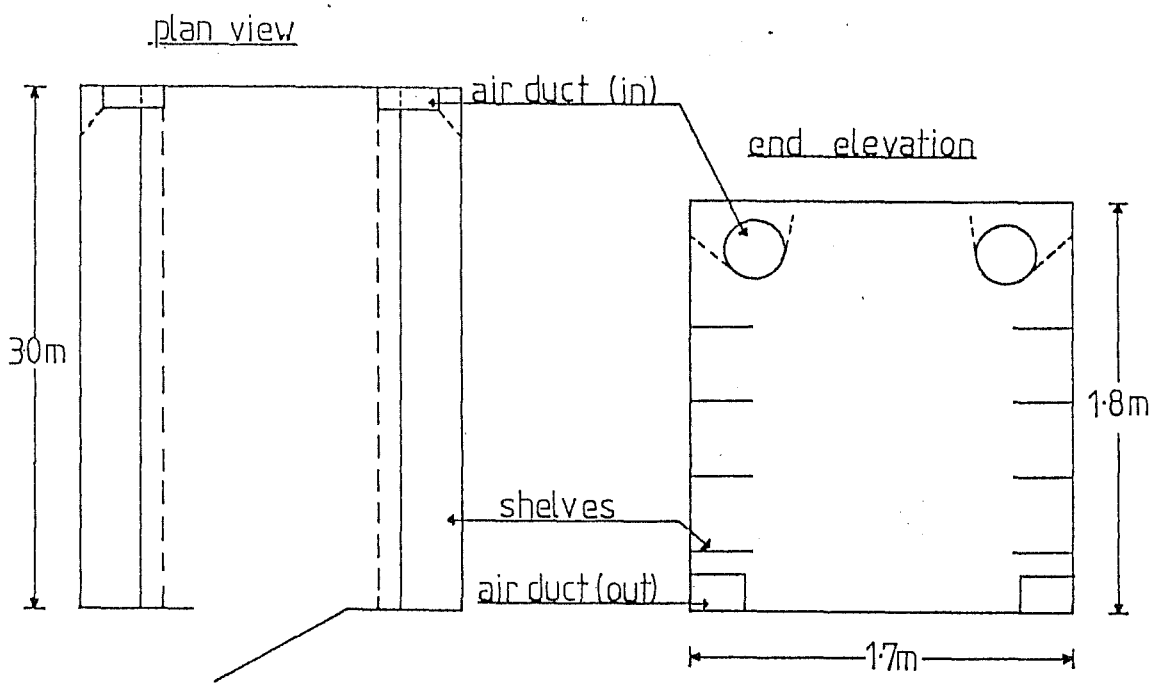
The major problem is that identically treated boxes can often produce very different yields. It is therfore important that sources of variation be identified so as they can either be eliminated or compensated for in the experimental design (Chanter 1976).

In 1984 a preliminary mushroom crop was grown in DSIR (Lincoln) mushroom growth cabinets, (see Fig 1.2). Although no results were recorded, the crop, nevertheless highlighted some obvious trends and sources of variation within the mushroom house. Most marked was a reduced yield from the boxes on the top shelves and particularily from the boxes at the door end of the cabinet. This was clearly the result of excessive drying caused by the design of the ducting system and was subsequently rectified prior to the cropping experiment.

Other areas where efforts were made to control known sources of unwanted variation were:

(1) The compost/spawn. An attempt was made to make the compost as uniform as possible by thorough mixing prior to box filling. The amount of compost used in each box was carefully weighed. A constant volume of spawn was used throughout and thoroughly mixed into the compost before

Fig 1.2. Plan of mushroom house, DSIR, Lincoln.



tamping the compost to a predetermined depth.

(2) The casing layer. Attention was paid to thorough mixing of the lime and to the amount of water applied, the depth of casing was also carefully checked to ensure uniformity.

(3) Picking. Experiments have shown this to be a major source of variability, (Cooke and Flegg 1962), hence an effort was made to pick all mushrooms at the same stage of development. The mushrooms were picked every day.

Other sources of variation that could not be eliminated or controlled such as enviromental differences within the house were compensated for through the utilization of a randomized block design. Randomized block designs, because of their ableness to remove unwanted variability have been recommended by several authors as the preferred experimental layout in mushroom trials (Lambert 1934, Cooke and Flegg 1962 and Chanter 1976). Randomized blocks also have the added advantage of leaving more degrees of freedom for error, thus permitting a more precise test of significance (Lambert 1934).

Lambert (1934) recommended the use of 5 or 6 replicates. In order to increase the experimental precision and also to balance the number of boxes in the house, each of the 9 treatments was replicated 8 times. The mushroom cabinets had 4 shelves on each of the 2 sides of the house, every shelf capable of holding 9 boxes, see Fig 1.2. The complete randomized block design, used to compensate for vertical and horizontal gradients, allowed 1 replication of each treatment

to be randomly assigned to each of the 8 shelves, (blocks).

The entire cropping experiment was conducted within one mushroom cabinet, thus the problems of associated with variation between houses was avoided.

Results of the cropping trial were subject to analysis of variance (ANOVA) using the statistical package, 'Teddy Bear'.

1.2.4b Compost

Compost was kindly supplied by Ashlin Mushrooms and had the following properties as determined by the Islington Freezing Works Laboratory, Christchurch.

Moisture	7Ø.5%
Dry matter	29.5%
Total N	1.95%
Ammonia	Ø.14%
pH	7.2

The composting procedure followed by Ashlin Mushrooms is a variation of the 'short method' described by Vedder (1978):

Day	
-13	Break bails of straw and add water.
-8	Turn straw and add water.
-5	Add horse manure and more water - mix.
-2	Turn and add water.
Ø	(Phase I). Stack, add chicken manure and turn
2	Turn stack, adding lime.
5	Turn stack, adding water.
6 or 7	(Phase II). Trays are filled and placed in peak heat house.

1.2.4c Spawn

Spawn was also kindly provided by Ashlin Mushrooms. A European strain, Ax6Ø, (Sinden-Hauser), was used and had been prepared by inoculation of wheat grains following the method of Stoller (1962).

1.2.4d Preperation of equipment

Wooden boxes with dimensions 35Ø mm x 25Ø mm x 15Ø mm deep were used as growth containers in which the mushrooms were grown. Prior to filling, the boxes were dipped in a 3% solution of 'Santobrite', (pentachlorophenol), to help preserve the timber and to kill any pests. The boxes were left for 24 h before filling with compost.

The mushroom growth cabinets were disinfected with a 3% formalin solution 1 week before use.

1.2.4e General procedure

(1) Spawn run. Eighty boxes were each filled with 3.3Kg of compost mixed with 25Øml of grain spawn, placed in the growth cabinets and covered with damp newspaper to prevent the surface compost from drying out. The newspaper was kept moist and the floors and walls wet by spraying water in the cabinet every day.

(2) Casing. After a 14 day spawn run, the boxes were cased with the appropriate casing material to a depth of 3Ø-4Ømm. The beds were again covered with damp newspaper to help prevent moisture loss and to maintain a high humidity level. (The day of casing equals day Ø).

(3) Ruffling. Eight days after casing the newspaper

was removed. On day 9, just before the first mushroom initials were formed, the casing layer was roughened. This was done to encourage a large and regular first flush and also to restore the structure of the casing material after the damaging effects of the frequent but necessary waterings.

(4) Watering. The water content of the compost throughout the spawn run was preserved by covering the boxes with damp newspaper; no water was applied directly to the compost. The water content of the casing materials was raised to field capacity in the first 3-4 days after casing. Water was applied lightly and frequently at a rate of approximately 1-2 litres/m^3/sprinkling. The optimum moisture level was estimated to be when water was easily squeezed from a handful af casing material. Field capacity was maintained by watering lightly every second day until just before pin initiation, (day 12). At this time watering was suspended until the mushrooms were approximately 5 mm in diameter, in order to avoid damaging the developing mushroom initials. A similar watering pattern was followed for subsequent flushes.

(5) Temperature. Throughout the spawn running phase, until 13 days after casing vegetative mycelial growth was encouraged by maintaining the bed temperature (ie. compost temperature) at 26 C $\pm$ 1 C. Heat produced by biological activity in the compost made it necessary to gradually decrease the air temperature from 26 C to 22 C in order to prevent the bed temperatures exceeding 27 C. During the first stages of pin formation, (day 13), the air temperature was

decreased to 16 C ± 1 C, the bed temperature consequently also dropped to between 16 and 18 C. These temperatures were maintained until the end of cropping.

(6) Ventilation. In commercial situations where mushrooms are grown in large boxes or trays, a great deal of heat is generated during spawn run. In these situations, ventilation with cool air during compost colonization is often necessary to prevent bed temperatures exceeding 28 C. Small boxes, as were used in this experiment, had insufficient compost volume to be able to generate much heat, hence it was not necessary to ventilate during spawning. This, to a certain extent, was an advantage as it meant high CO_2 levels were maintained, which in turn encouraged good vegetative mycelial growth.

A rapid decrease in the CO_2 level plus temperature drop are key factors in aiding the development of fruiting bodies. Formation of tiny mycelial aggregates on the surface of the casing layer on days 12 and 13 provided the means of recognizing the correct time to ventilate with fresh air which was commenced on day 13 (at the same time as the temperature drop) and continued throughout the cropping period.

(7) Humidity. Relative humidity was kept as high as was possible (without using humidifiers) for the entire cropping trial by ensuring the floor and walls were kept wet. The relative humidity was maintained at a fairly constant 90%, however after day 13, when ventilation commenced, the relative humidity fluctuated between 80% and 90% in response

to the heating and cooling of the ventilating unit. Relative humidity was measured using a thermohydrometer.

(8) Harvesting. Mushrooms were harvested at the point where the veil was just beginning to rupture (ie. in between the button stage, (cap tightly closed) and the cup stage, (veil broken)). Removal of the mushrooms from the beds was effected with a gentle twisting motion. If there was any danger of damaging the surrounding mushroom initials using this technique the sporocarps were trimmed off with a knife. Before weighing, any adhering casing material at the base of the mushroom was removed with a sharp knife.

1.2.4f Water content of the sporocarps

The average moisture content of the mushrooms was determined by freeze drying weighed mushrooms for 48 h. After this time the mushrooms were reweighed and placed back in the freeze dryer for a further 24 h. At the end of this time the sporocarps were again weighed to ensure that all water had been removed.

1.3 RESULTS AND DISCUSSION

1.3.1 PHYSICAL AND CHEMICAL PROPERTIES

1.3.1a pH and buffering capacity

The pH of the unlimed casing materials ranged from near neutral for Pu and PuFm (pH 6.8) to extremely acid (less than pH 4.5) for Pe. Addition of lime raised the pH of all materials to between 7.4 and 7.5 (Fig 1.3).

Table 1.1 shows the ability of the 9 casing materials to resist pH change. After the addition of 2ml of Ø.1N HCl all casing materials without lime, with the exception of Pu showed a decrease in pH of between Ø.3 and Ø.4 pH units. Pu decreased by Ø.6 pH units while distilled water with no buffering capacity decreased from pH 6.7 to pH 2.7. Addition of lime to the materials in most instances slighty improved the already high buffering capacities.

Results from the periodic measurement of pH over the duration of the cropping trial indicated that the buffering capacity of all limed casing materials was sufficent to counter the acids produced during the growth of A. bisporus. The pH of all the limed casing materials was within Ø.2 pH units of pH 7.5 after 48 days.

Large buffering capacities are to be expected from these materials as all, with the exception of Pu and PuFm, consist of nearly 1ØØ% organic matter. Organic matter is well known for its buffering properties; a result of the large number of positive and negative exchange sites on its surfaces. Pu,

Fig 1.3. pH of casing materials with and without lime.

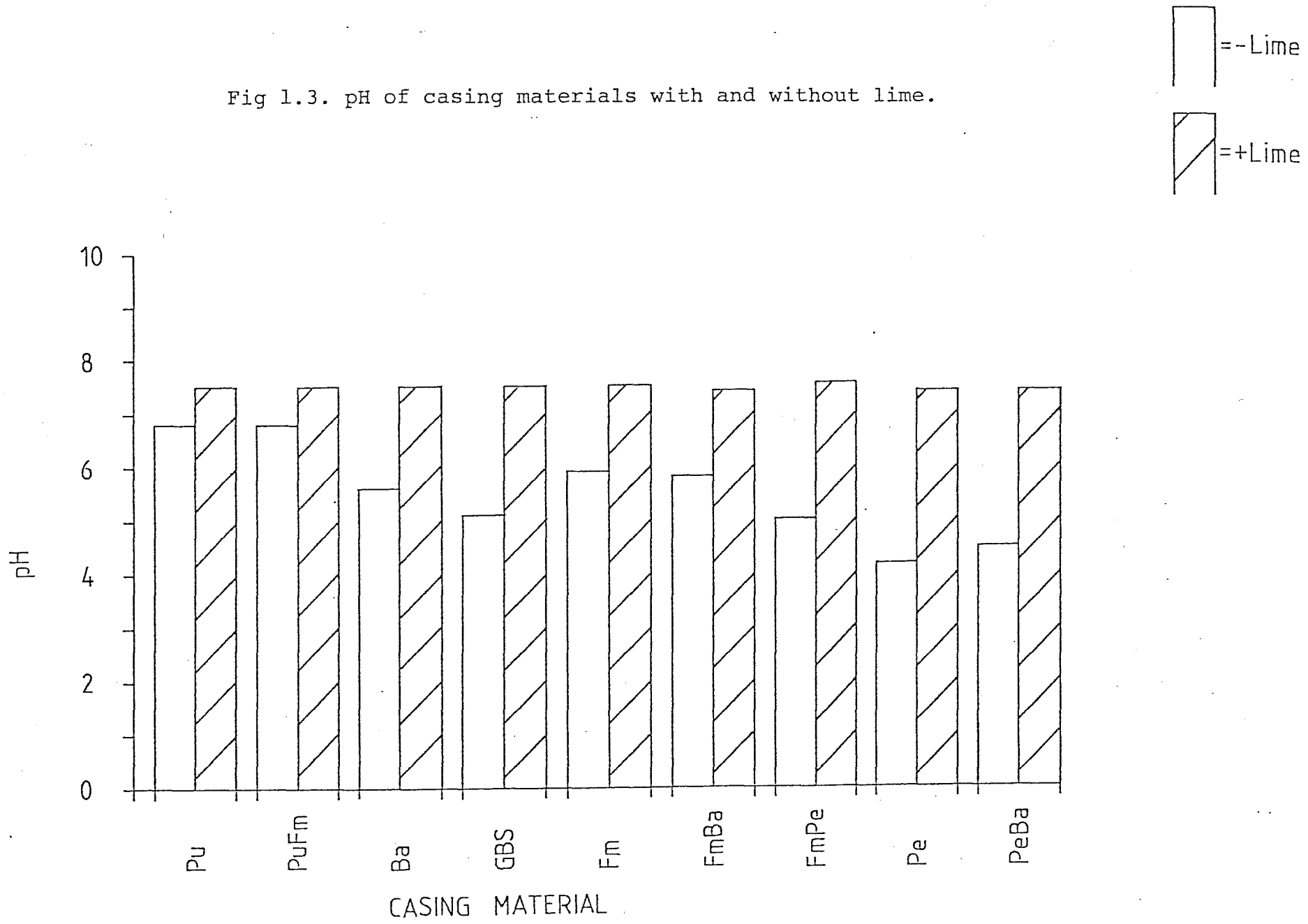


Table 1.1. Buffering capacity of casing materials, with and without lime.

	pH					
CASING	-lime	+1ml Ø.1N HCl	+2ml Ø.1N HCl	+lime	+1ml Ø.1N HCl	+2ml Ø.1N HCl
Pu	6.8	6.6	6.2	7.5	7.3	7.1
PuFm	6.8	6.6	6.4	7.5	7.3	7.1
Ba	5.6	5.4	5.2	7.5	7.3	7.2
GBS	5.1	4.8	4.5	7.5	7.3	7.1
Fm	5.9	5.7	5.5	7.5	7.5	7.3
FmBa	5.8	5.7	5.5	7.4	7.4	7.3
FmPe	5.Ø	4.8	4.6	7.5	7.3	7.2
Pe	4.2	4.1	3.8	7.4	7.3	7.3
PeBa	4.5	4.4	4.2	7.4	7.2	7.1
Water	6.7	3.7	2.7	-	-	-

while containing very low amounts of organic matter still has a large buffering capacity, because of to the surface charges on the fine clay particles (Metson and Blakemore 1968).

1.3.1b Electrical conductivity

Electrical conductivity provides a measure of soluble salts in the casing material. Fig 1.4 shows the electrical conductivity of the different casing materials. No obvious trends are present and the effect of adding lime is unclear. In some cases, after the addition of lime, the levels of soluble salts increased, eg. Ba and GBS, while in others, eg. Fm and FmPe, the levels of soluble salts were suppressed.

Electrical conductivity was found to increase in all instances when measured over the duration of the cropping trial (Fig 1.5). Flegg (1961), Yeo and Hayes (1979), Hayes (1981) and others have also reported this trend.

1.3.1c Exchangeable cations

(1) Cation exchange capacity (CEC). Cation exchange capacity provides a measure of the freely available cations in the casing material. Cation exchange in soils is a reversible chemical reaction which takes place between the cations held on the surface of soil minerals, clay colloids, organic matter and the cations in salt solutions and acids (Chapman 1965). Fig 1.6 shows the results from the cation exchange determination. Pu and PuFm have the lowest CEC indicating the lower number of cation exchange sites and hence the lower nutrient status of the medium. These lower values are due primarily to the lack of organic matter. Pu,

Fig 1.4. Electrical conductivity of casing materials with and without lime.

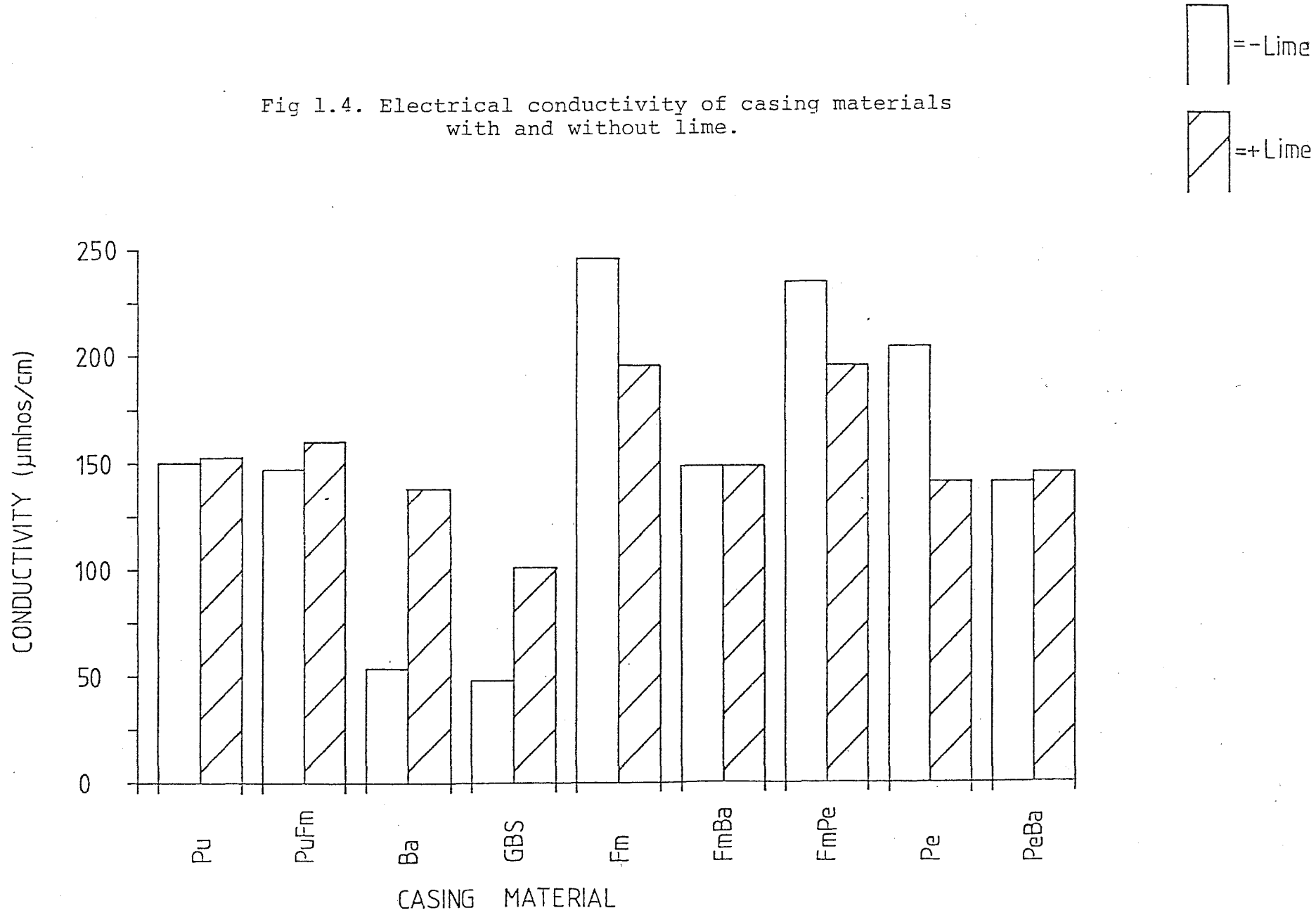


Fig 1.5. Change in electrical conductivity over time, from casing through to the end of cropping.

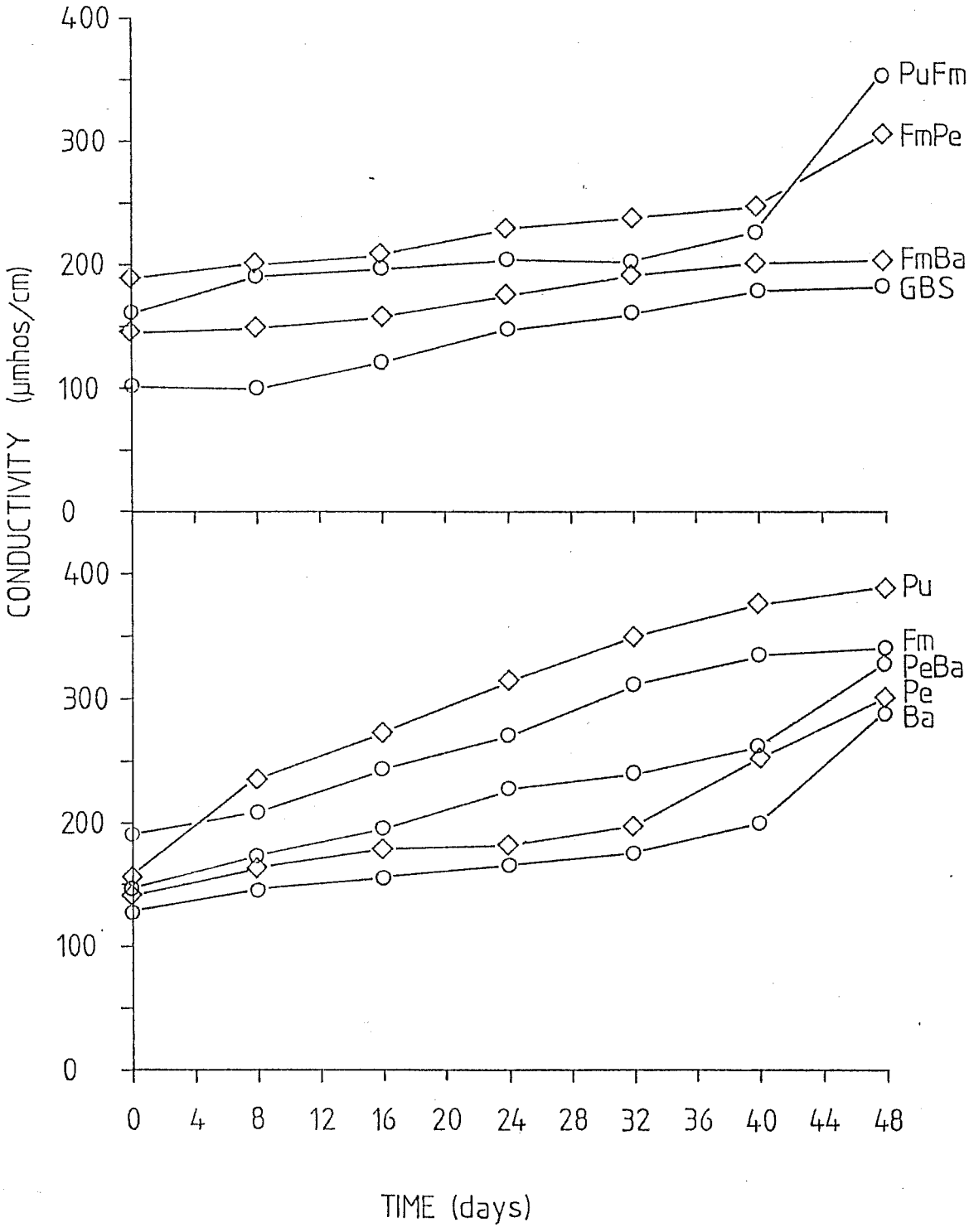
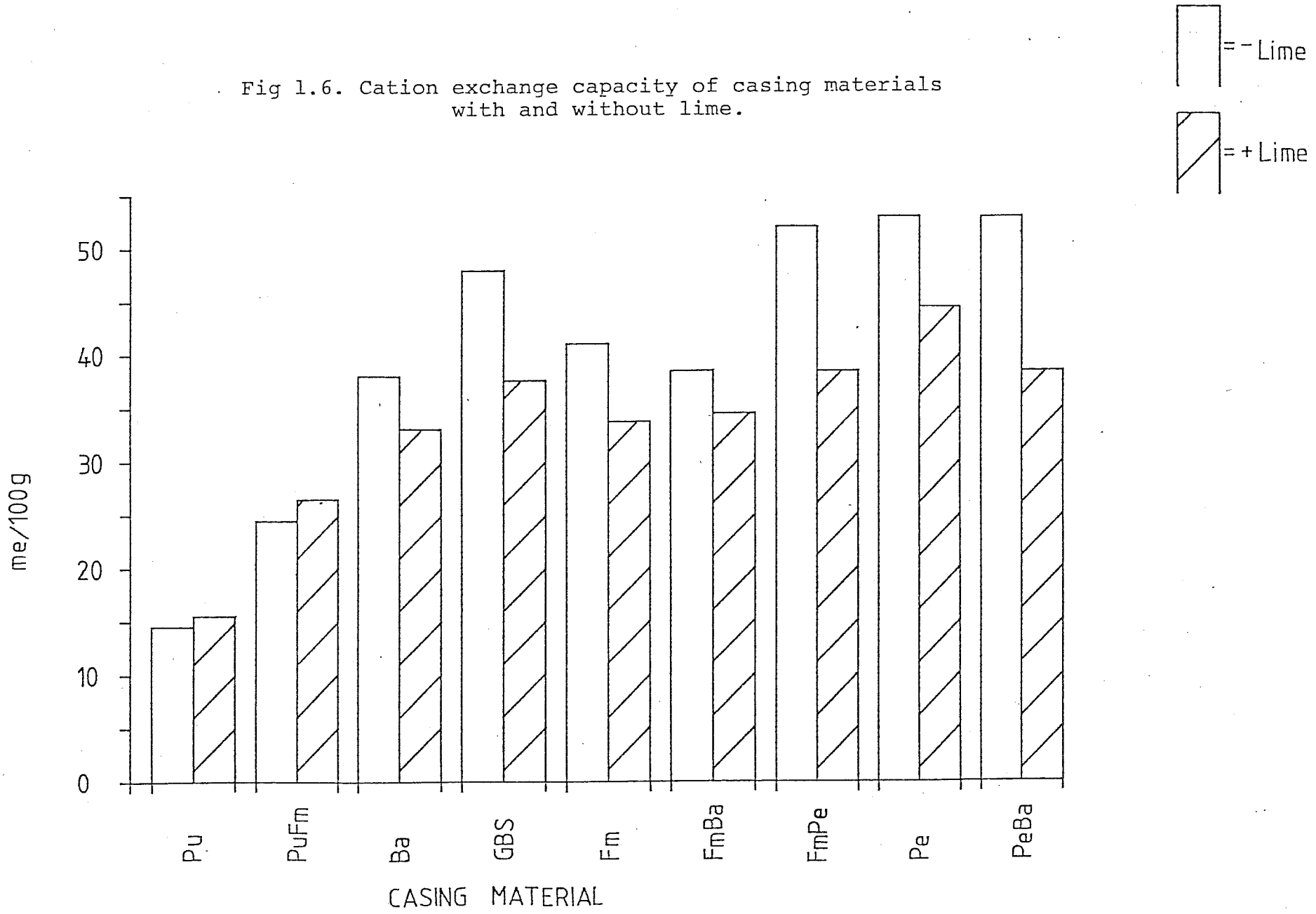


Fig 1.6. Cation exchange capacity of casing materials with and without lime.



however, posesses a large proportion of clay colloids and porous aggregates which provide a large surface area of exposed charges. As a result, Pu does have many exchange sites. This is further reflected by the fact that the calculated CEC for Pu (14.5 me%) falls within the 'medium' CEC category of New Zealand soils (Metson 1956). The CEC's of the other materials analysed, fall within either the 'high' or 'very high' categories as would be expected of organic mediums.

All the casing materials responded negatively to the addition of lime with the exception of Pu and PuFm which increased slightly.

(2) Total exchangeable bases (TEB). Total exchangeable bases refers to the sum of the metal cations extracted by NH_4COOCH_3 (Chapman 1965). Tables 1.2 and 1.3 provide a list of the extracted cations. The quantities of exchangeable cations with the exception of Ca^{2+} (because of added $CaCO_3$) are very similar in both the limed and unlimed casing materials.

For each material TEB results are slightly under estimated, this is because exchangeable Na^+ was not measured.

(3) Percentage base saturation. Percentage base saturation signifies the proportion of the metal cations relative to the total CEC of the medium (Metson and Blakemore 1968). The limed casing materials, as a result of the high lime levels are 100% base saturated; any deficit between CEC and TEB is filled by the excess of exchangeable Ca^{2+} in the casing material. In contrast, the unlimed casing materials

Table 1.2. Exchangeable cations, total exchangeable bases and percentage base saturation of casing materials with lime.

CASING	Ca	Mg	K	Al	Fe	Mn	TEB	%BS
Pu	15.1	Ø.18	Ø.33	Ø.Ø2	Ø.Ø	Ø.ØØ2	15.6	1ØØ%
PuFm	25.7	Ø.3Ø	Ø.39	Ø.Ø	Ø.Ø	Ø.ØØ7	26.4	1ØØ%
Ba	3Ø.8	Ø.72	1.28	Ø.Ø	Ø.ØØ2	Ø.Ø2	32.8	1ØØ%
GBS	35.6	Ø.86	1.14	Ø.Ø	Ø.Ø	Ø.Ø4	37.6	1ØØ%
Fm	32.4	Ø.51	Ø.79	Ø.Ø	Ø.Ø	Ø.Ø2	33.7	1ØØ%
FmBa	32.6	Ø.63	1.Ø7	Ø.Ø	Ø.Ø	Ø.Ø2	34.6	1ØØ%
FmPe	36.5	1.11	Ø.73	Ø.Ø	Ø.Ø	Ø.Ø2	38.4	1ØØ%
Pe	41.4	2.2Ø	Ø.75	Ø.Ø	Ø.Ø	Ø.ØØ9	44.4	1ØØ%
PeBa	36.2	1.3Ø	1.Ø4	Ø.Ø	Ø.Ø	Ø.Ø15	38.6	1ØØ%

Table 1.3. Exchangeable cations, total exchangeable bases and percentage base saturation of casing materials without lime.

CASING	Ca	Mg	K	Al	Fe	Mn	TEB	%BS
Pu	2.15	Ø.Ø9	Ø.Ø4	Ø.Ø	Ø.Ø	Ø.Ø	2.3	16%
PuFm	2.1Ø	Ø.23	Ø.25	Ø.Ø	Ø.Ø	Ø.Ø1	2.6	11%
Ba	1.7Ø	Ø.75	1.54	Ø.Ø	Ø.Ø	Ø.Ø4	4.Ø	11%
GBS	1.88	Ø.88	1.25	Ø.Ø	Ø.Ø	Ø.Ø7	4.1	9%
Fm	1.73	Ø.51	Ø.74	Ø.Ø	Ø.Ø	Ø.Ø4	3.Ø	7%
FmBa	1.8Ø	Ø.63	1.1Ø	Ø.Ø	Ø.Ø	Ø.Ø4	3.6	9%
FmPe	1.8Ø	1.58	Ø.51	Ø.Ø	Ø.Ø	Ø.Ø2	3.9	8%
Pe	2.4Ø	2.Ø6	Ø.18	Ø.Ø	Ø.Ø	Ø.ØØ8	4.7	9%
PeBa	1.8Ø	1.81	Ø.87	Ø.Ø	Ø.Ø	Ø.Ø2	4.5	9%

All values are in me/1ØØg oven-dry casing material

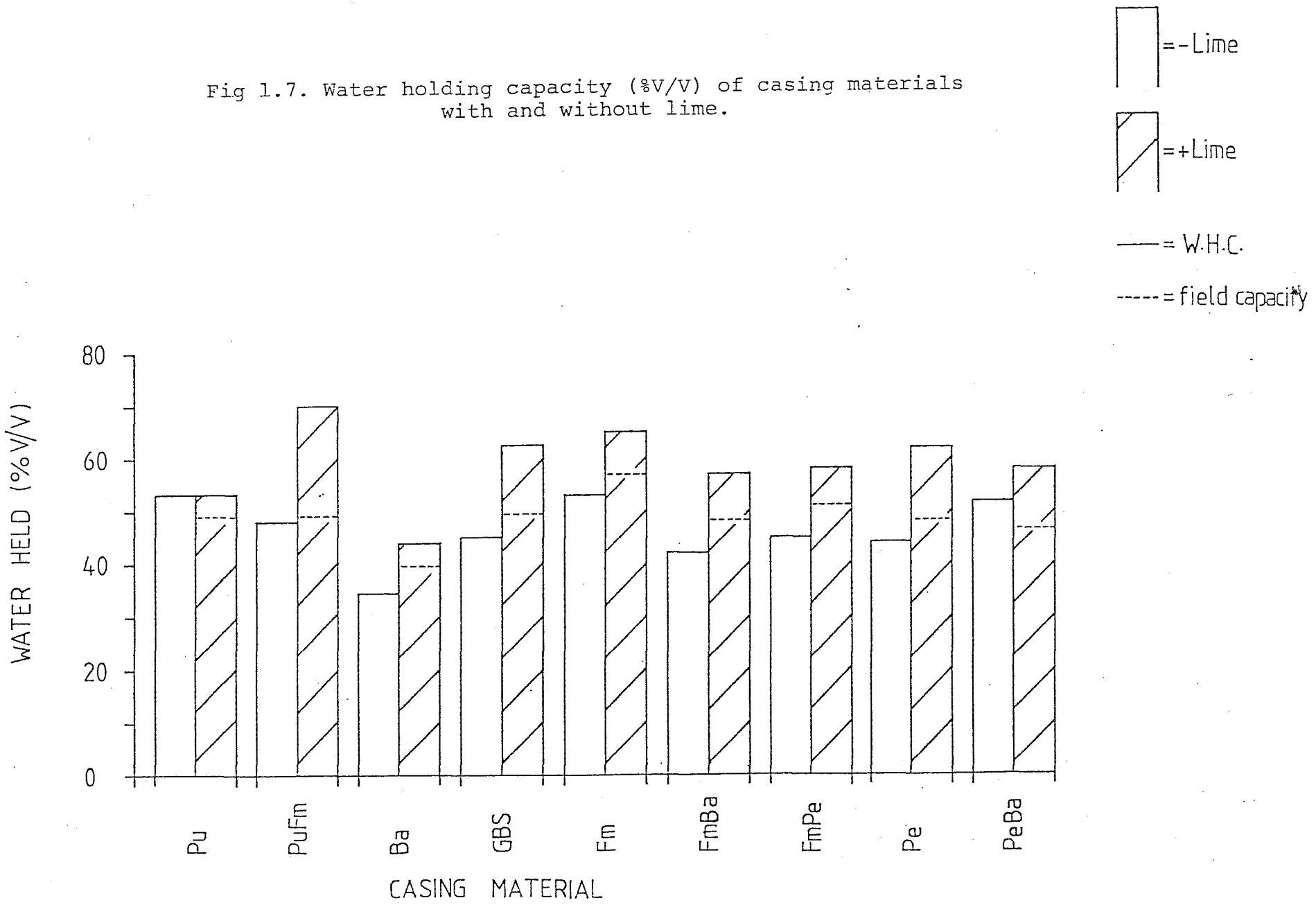
have low %BS values. This is most likely a result of the low pH's of the unlimed casing materials reducing the availability of the majority of the metal cations (Taylor and Pohlen 1962). The large difference between TEB and CEC in this case is due to exchangeable H^+ (exchange acidity) (White 1979).

1.3.1d Water holding properties

The water holding capacities (%V/V) of the 9 casing materials are expressed in figure 1.7 (reference should be made to Appendix 2 for water holding capacities on a weight basis). The water holding capacities of the unlimed casing materials range from 35% (V/V) for Ba, to 53% (V/V) for both Pu and Fm. Addition of lime to the casing materials increased their water holding capacities by between 10% and 20% (V/V) in all instances except Pu, where there was no change.

Field capacity, ie. the maximum amount of water held by a soil after drainage, provides a more realistic measure of water retention than does water holding capacity, ie. the amount of water required to saturate a soil. An estimate of the field capacities of the limed casing materials, as used in the cropping trial, are given in fig 1.7. The results reflect the amount of water held by the individual casing materials 10 days after casing. At this particular time the casing layer should have ideally been at field capacity. It appears from the results that this was the case; the water content of all the materials, except PuFm, was between 80% and 90% of their water holding capacities, a water content

Fig 1.7. Water holding capacity (%V/V) of casing materials with and without lime.



which is to be expected from materials high in organic matter or porous pumice (pers. comm. J.A. Adams, Soil Science Dept., Lincoln College).

1.3.1e Density

Both bulk density and particle density of the 9 casing materials are represented in Fig 1.8.

McDonald (1962), suggested a range of bulk density classes for classifying New Zealand soils, two of which are:

(1) Less than Ø.2gcm^{-3}, (very low), typical of peats

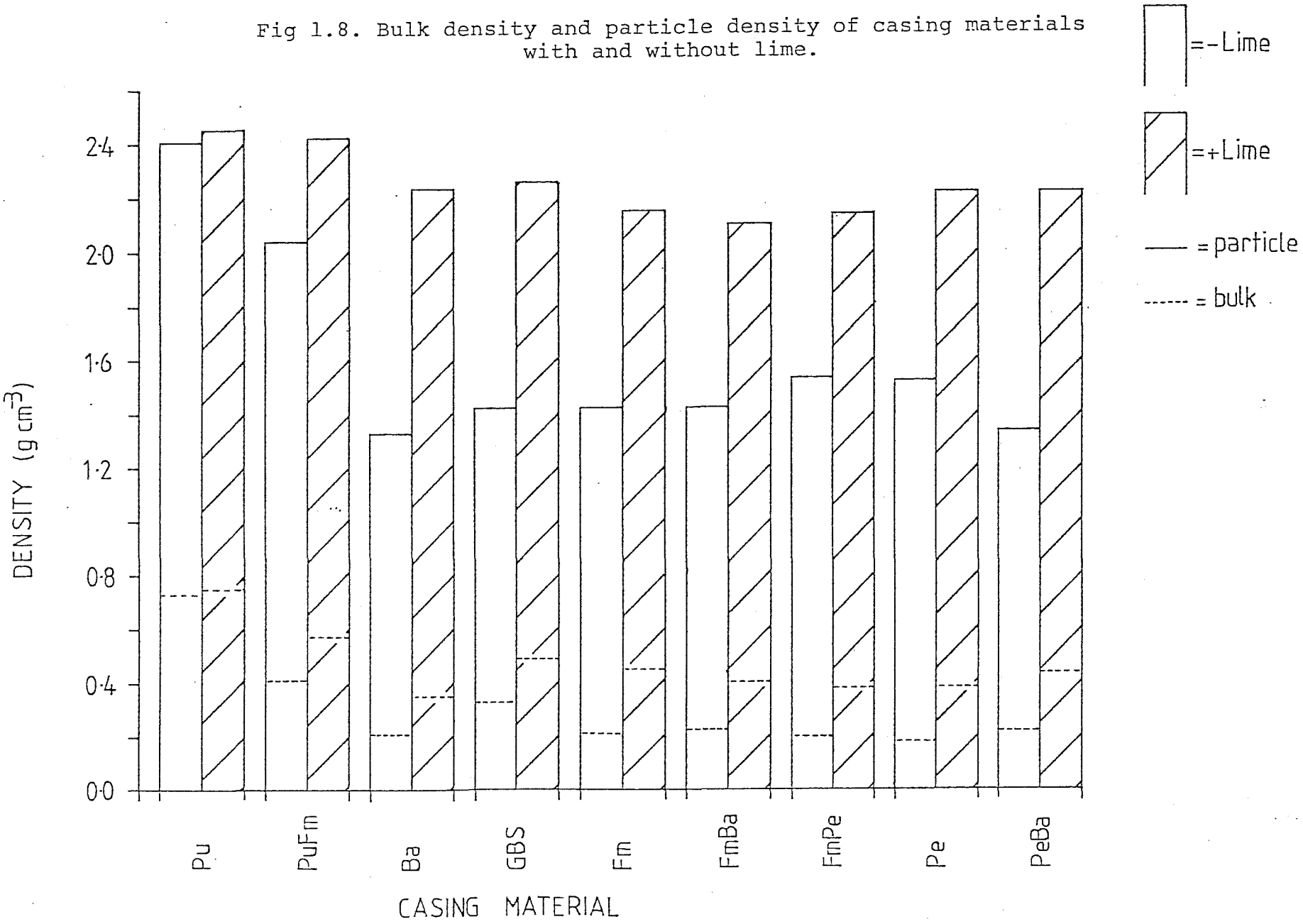
(2) Between Ø.2 and Ø.8gcm^{-3}, (low), typical of yellow-browm pumice soils. Examination of Fig 1.8 shows that these results correlate well with the above categories. Fig 1.8 also reveals that addition of lime approximately doubled the bulk densities of the casing materials; Pu, however is once again the exception. Addition of lime to Pu only very slightly increased its bulk density indicating that Pu consists of a large proportion of fine particles, similar in size to those of lime, ie. within the clay, silt, size range of less than Ø.Ø2mm. Thus adding lime to Pu was like adding Pu to itself and consequently the bulk density remained more-or-less unchanged.

The particle densities of the casing materials behaved in a similar manner to the bulk densities following the addition of lime for reasons which mimic those described above.

1.3.1f Porosity

Bulk density in particular, but also particle density,

Fig 1.8. Bulk density and particle density of casing materials with and without lime.



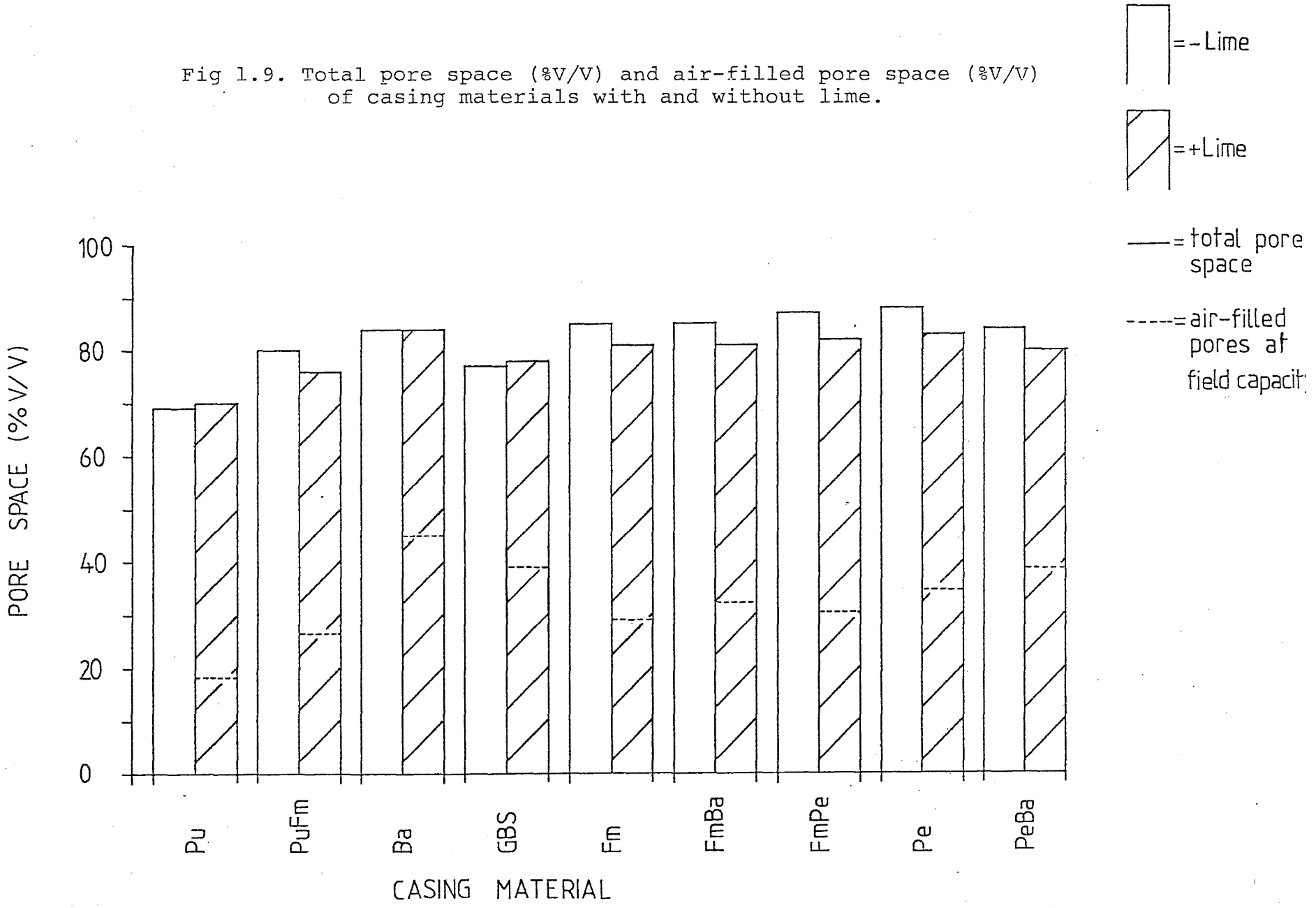
provides a measure of soil structure (Blake 1965). This is reflected most clearly by an examination of porosity, which is itself a function of both particle and bulk density.

The increase in particle and bulk density, which in the majority of cases follows the addition of lime, leads to a decrease in total pore space, see Fig 1.9. The total pore space of the two bark products, Ba and GBS, however, did not change after addition of lime. The most likely explanation for this apparent anomaly relies on the fact that the large bark chips (10mm max. chip size) created large diameter pores which as result of their size remained relatively unaffected by the addition of lime.

Examination of the percentage volume of air filled pores at field capacity shows Ba to have the greatest volume (45%) and Pu the smallest (18%) (Fig 1.9). These results are a direct reflection on the pore sizes of the casing materials. Theoretically, pores with a diameter of less than 0.075mm (micropores) are not free draining (Taylor and Pohlen 1962). This implies that a dense medium with a majority of micropores, for example Pu, will hold a large proportion of water, relative to air. Ba, however, because of its large particle size has many pores larger than 0.075mm diameter and consequently these large pores will always drain free of water leaving the medium with a large proportion of air relative to water.

It should be pointed out that the % volume of air-filled pores plus the % volume of water at field capacity, should equal % total porosity. The fact that it does, (Table 1.4),

Fig 1.9. Total pore space (%V/V) and air-filled pore space (%V/V) of casing materials with and without lime.



adds considerable weight to the validity of these results.

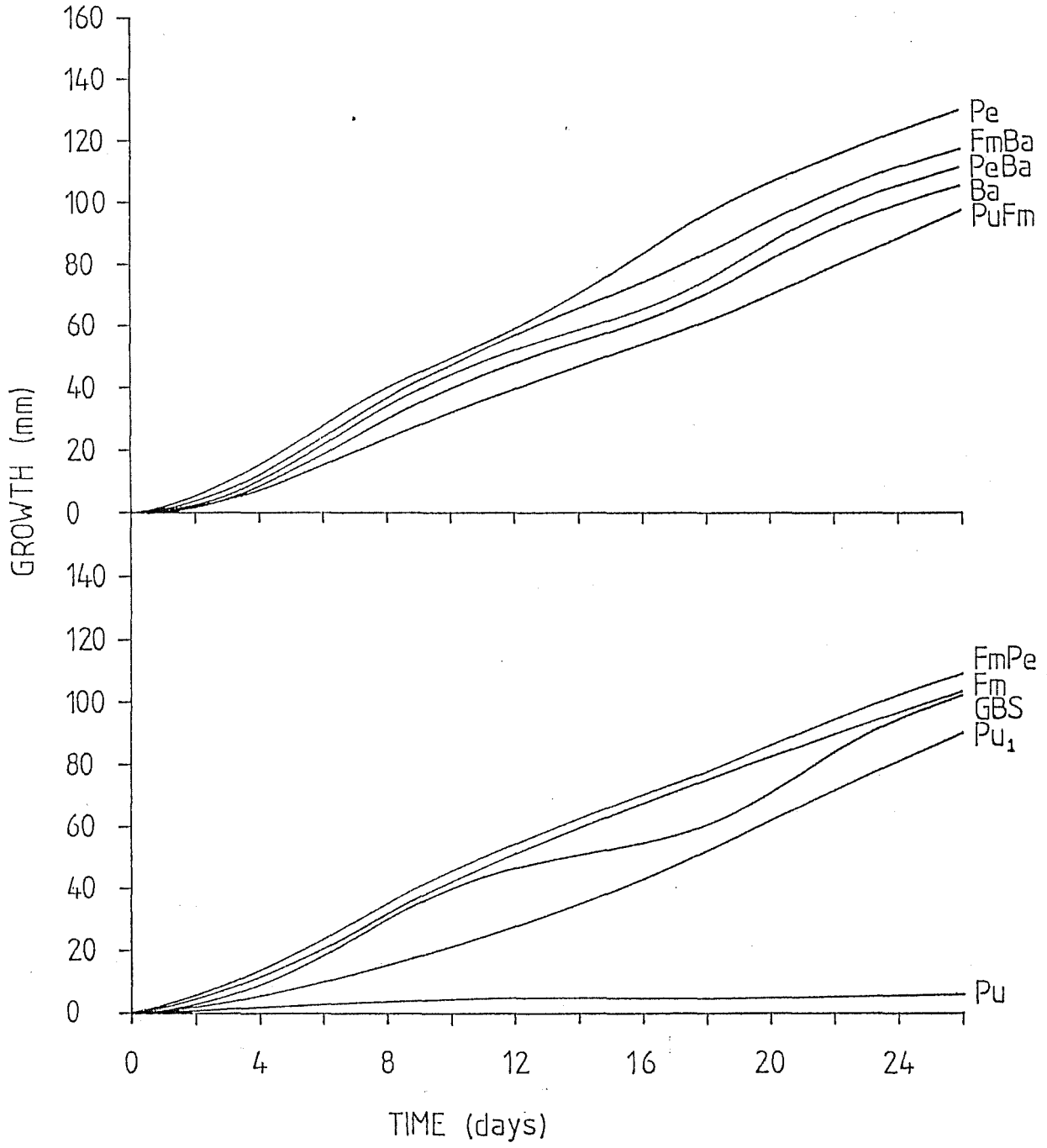
1.3.2 THE EFFECT OF THE CASING MATERIAL ON MYCELIAL MASS AND RATE OF MYCELIAL GROWTH

1.3.2a Rate of growth of mushroom mycelium through the casing

Fig 1.1Ø illustrates the growth rates of mushroom mycelium through the 9 casing materials. The growth rates of the mycelium were similar in all instances except Pu where the mycelium grew no more than 5mm in approximately 1Ø days. Examination of the physical and chemical properties of Pu revealed that the most likely reason for this was the small percentage of air filled pores in the casing material. To test this hypothesis, Pu was washed through a Ø.2mm sieve, the filtered fraction discarded and the growth rate experiment re-run. The results of the re-run are also given in Fig 1.1Ø where the 'filtered' Pu = Pu_1. The performance of Pu_1 strongly indicated that the lack of air filled pores, (ie. a reflection on the bulk density and consequently the structure of Pu), was the major reason for the poor performance of Pu.

After 26 days the mycelial growth rates had not begun to decline as would be expected considering there was no compost or nutrient rich media in the glass tubes raiseing questions as to the source of nutrients maintaining growth. For interest the 'growth rate tubes' were not discarded after the 26 day period of the original experiment. Observation after 4Ø days showed that the mycelium had continued to grow,

Fig 1.10. Growth of mushroom mycelium through casing materials.



Pu_1 = Pu with particles < 0·2 mm removed

in most instances reaching a height of more than 200mm. Primordia were even formed inside some tubes. It seems unlikely that 5 g of grain spawn could supply all the nutrients necessary for this growth. In addition, Pu_1, even though possessing more favourable physical characteristics than Pu, still did not perform as well as the organic casing materials. The lower CEC, and hence nutrient status of Pu, when compared with the other casing materials, was a likely contributing factor. These facts tend to suggest that the casing layer, although not, nor intending to be nutrient rich does contribute to the nutrition of A. bisporus.

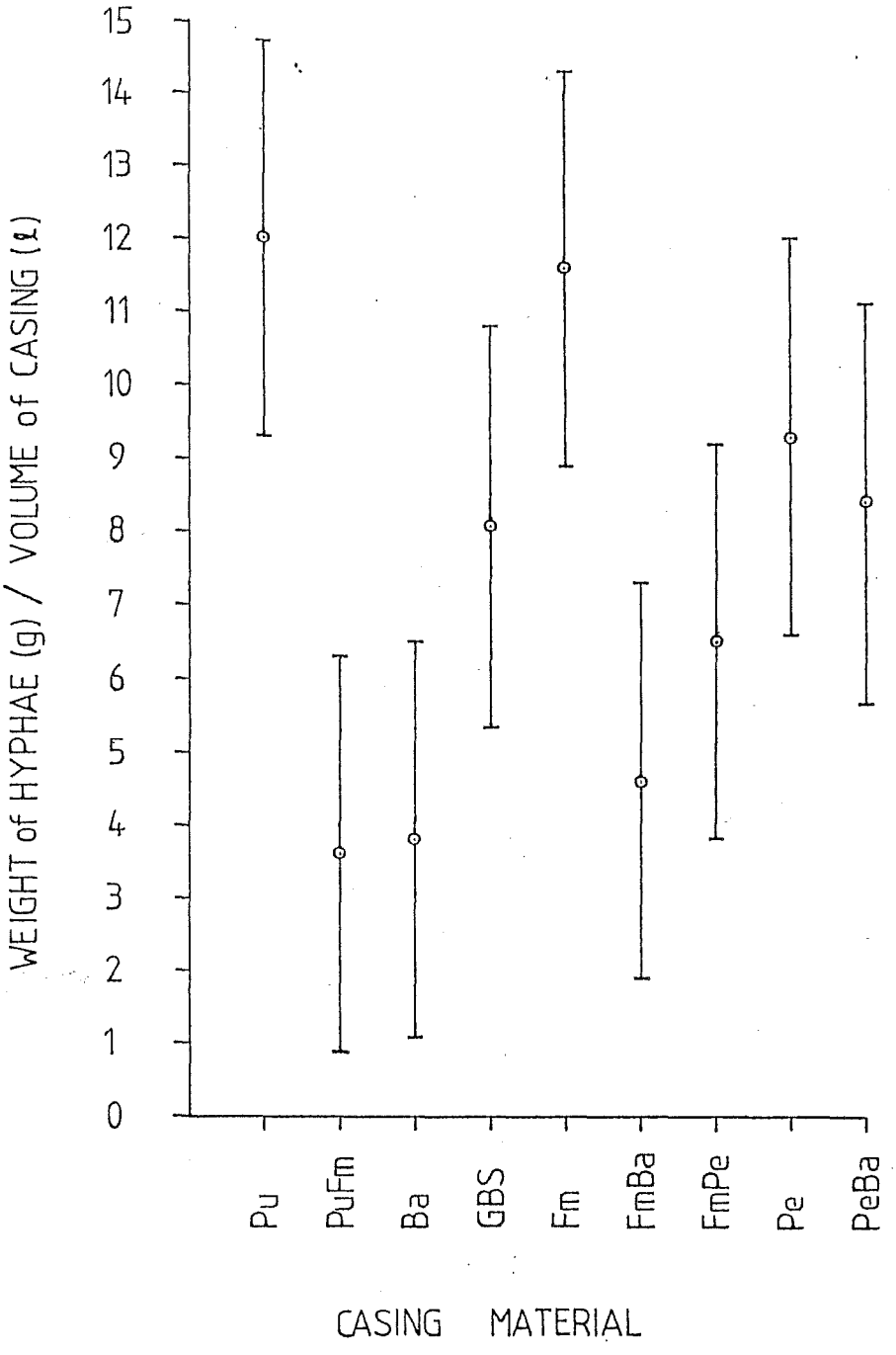
1.3.2b Mycelial mass in the casing layer

Statistical analysis (ANOVA and Newman Keuls test for unplanned comparisons) of the results represented in Fig 1.11, revealed no significant difference between the masses of mycelial strands present in the different casing materials. Further analysis showed that there was no significant correlation between yield and mycelial mass in the casing layer.

Examination of the standard error bars in Fig 1.11 gives an indication of the amount of variability within treatments. It appears that four replicates were not sufficient to account for the unexpectedly large variability encountered within each sampling unit (box). This variability suggests that the mass of mycelium in the casing layer is not an important factor influencing final yield.

It is possible that the number and length of mycelial

Fig 1.11. Mycelial mass in the casing layer.



strands growing through the casing layer may have correlated more significantly with yield than mass, since it was seen that the diameter and hence the mass of similar length mycelial strands varied between treatments. Mycelial strands taken from Pu were usually 1-2mm in diameter, while strands removed from Ba were usually finer (< Ø.75mm) and therefore lighter. A possible explanation for these results is again related to the stucture of the casing materials. A dense casing medium, such as Pu would have retarded mycelial penetration of the casing layer. A lighter medium such as Ba, would presumably provide less resistance. Correlation between mycelial mass and bulk density is not statistically significant, however, the correlation co-efficient, (r = Ø.62), does suggest a relationship between these two factors.

Plant physiological studies have shown that epicotyls of pea seedlings, when confronted with an obstruction increase their production of ethylene which in turn causes radial swelling of the epicotyl, allowing greater force to be exerted on the obstruction (Goeschl et al. 1966). Ethylene production has been reported in A. bisporus (Lockard and Kneebone 1962) and recently Wood and Hammond (1977) and Ward et al. (1977) demonstrated ethylene production by the mycelium. Although Ward et al. (1977) concluded that there was no evidence for a regulatory role of ethylene in the growth or development of A. bisporus there remains the possibility that the heavy mycelial strands found in Pu may result from the production of ethylene in response to stress,

in a manner similar to that in plants.

1.3.3 CROPPING TRIAL

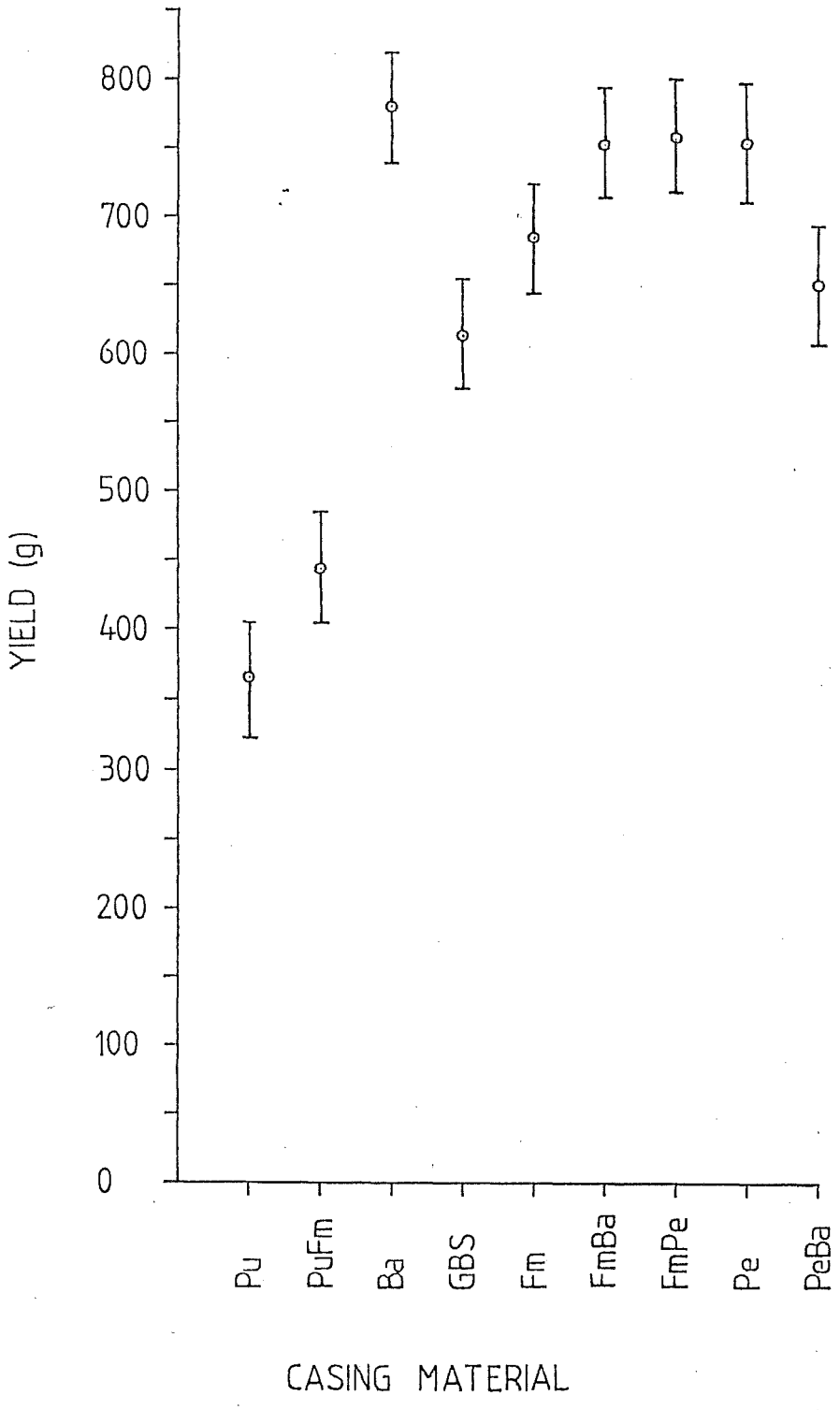
1.3.3a Cropping performance of the nine casing materials

The mean yield of mushrooms over 3 flushes is illustrated in Fig 1.12. Analysis of variance revealed a significant difference between treatments ($P < Ø.ØØ1$). Duncan's New Multiple Range Test for unplanned comparisons was used to highlight differences between treatments. Pu and PuFm differed significantly from all other casing materials ($P < Ø.Ø5$). GBS was significantly different to Ba, FmBa, FmPe and Pe ($P < Ø.Ø5$), but did not differ significantly from either Fm or PeBa. Generally the first, second and third breaks in a mushroom crop are the largest with total production decreasing in the fourth and subsequent flushes (Vedder 1978). The mushroom trial conducted in the growth chambers at Lincoln did not show this usual trend. The first flush was unexpectedly large, while the second and third flushes produced yields substantially less than expected. For the following reasons compost quality appears to be the most likely explanation for these results:

(1) 'Ashlin Mushroom's' compost is often of poor nutritive quality and the crops it supports are seldom larger than those produced in the cropping trial (pers. comm. F.R. Sanderson DSIR, Lincoln).

(2) The cropping trial results are well explained in terms of a nutrient deficient compost; the large first flush

Fig 1.12. Mean yield of mushrooms; total over 3 flushes.



(a direct result of the extra measures taken to ensure good compost colonization) would have used a major portion of the available nutrients, leaving only a small reserve for subsequent flushes.

Disease could also have been responsible for the decreased yield but for several reasons this was dissmissed.

(1) The decreased yield, if caused by disease would most likely be confined to a small number of boxes and not to every box as was the case in the cropping trial.

(2) A number of deformed mushrooms were noticed and were investigated for signs of pathogens. Sections were examined under the transmission electron microscope but no evidence was found to suggest the deformities were the result of pathogen attack.

(3) Nematodes are often responsible for the collapse of mushroom crops (pers. com. F.R. Sanderson, DSIR, Lincoln). A small number of nematodes were found in some boxes towards the end of cropping, however, it was unlikely that they alone were the cause of the reduced second and third breaks.

Despite this problem the yields produced by the more productive casing materials, (Ø.75Ø kg mushrooms / kg dry compost and greater), compare favourably with published results from cropping trials (Edwards 1974 and Hayes 1981). Cooke and Flegg (1962) indicated that there was little value to be gained by cropping experimental trials for longer that 28 days. They showed that after this period an acceptable percentage standard error, (12.6%), was reached. The trial conducted at Lincoln was cropped for 26 days, (3 flushes) and

the percentage standard error at the end of this time was 6.6%.

The nutrient poor compost, as well as decreasing yield in flush 2 and 3 also appeared to reduce the differences between treatments especially in flush 3. In order to gain an indication of the trends within each break and also the effect of the compost on yield, the results of each flush were analysed separately (Fig 1.13, 1.14 and 1.15). For the purpose of this analysis a flush was defined as the 5 day period encompassing the peaks of production. Fortunately the flushes from each treatment coincided and were well defined, however, in some instances there was a small and unavoidable production of mushrooms between flushes; these mushrooms were disregarded. Fig 1.15 shows clearly the predicted 'smoothing effect' in the third flush. Statistical analysis, (ANOVA and Duncan's New Multiple Range Test), of this flush revealed no significant difference between treatments. Results of the ANOVA of flush 1 showed a significant difference between treatments ($P < Ø.ØØ1$). Duncan's New Multiple Range Test revealed 3 significantly different groups ($P < Ø.Ø5$); (1) Pu, PuFm and Fm (2) GBS (3) Ba, FmBa, FmPe, Pe and PeBa. Results from the ANOVA of flush 2 revealed the differences between treatments to be significant, ($P < Ø.ØØ1$), while Duncan's New Multiple Range Test highlighted 2 significantly different groups ($P < Ø.Ø5$); (1) Pu and PuFm (2) Ba, GBS, Fm, FmBa, FmPe, Pe, PeBa. Analysis of the individual flushes, provides a more detailed picture of events and shows a declining treatment effect as a result of the poor compost. The overall

Fig 1.13. Mean yield of mushrooms from flush 1.

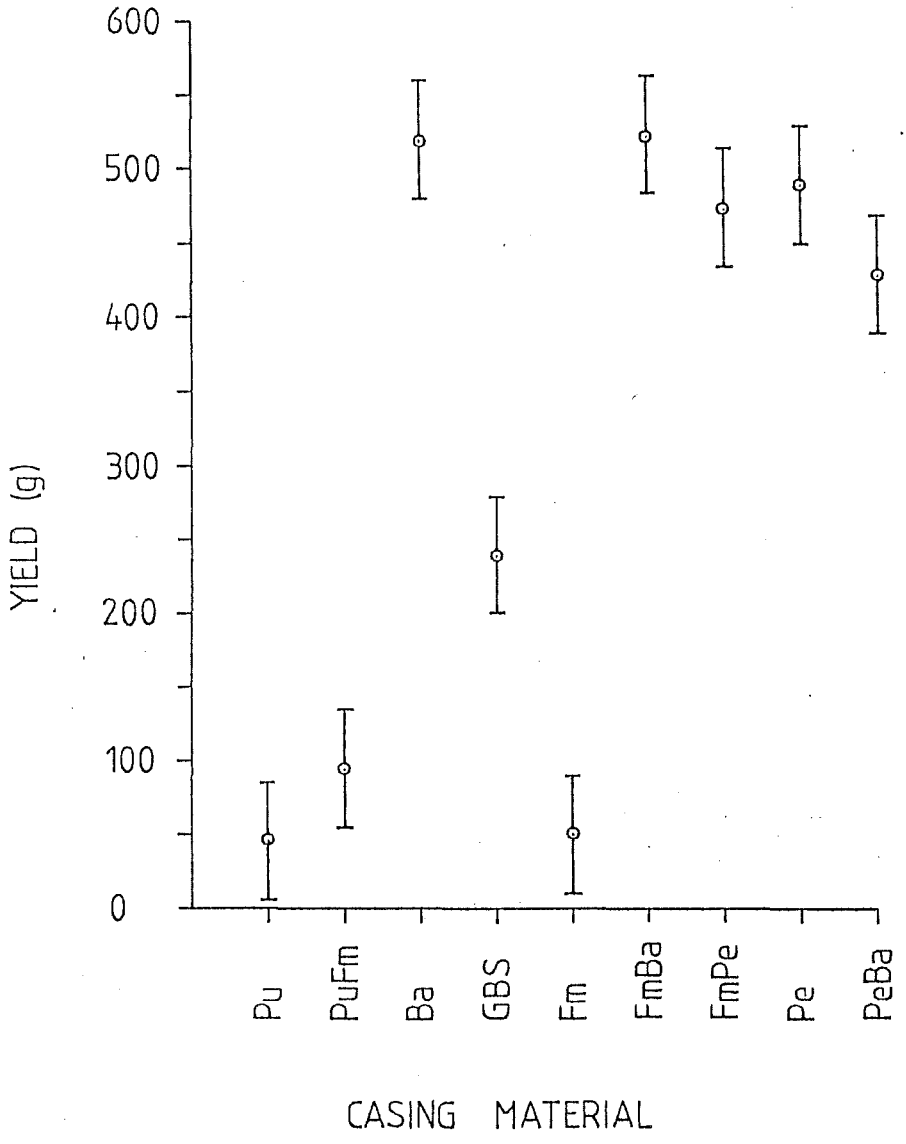


Fig 1.14. Mean yield of mushrooms from flush 2.

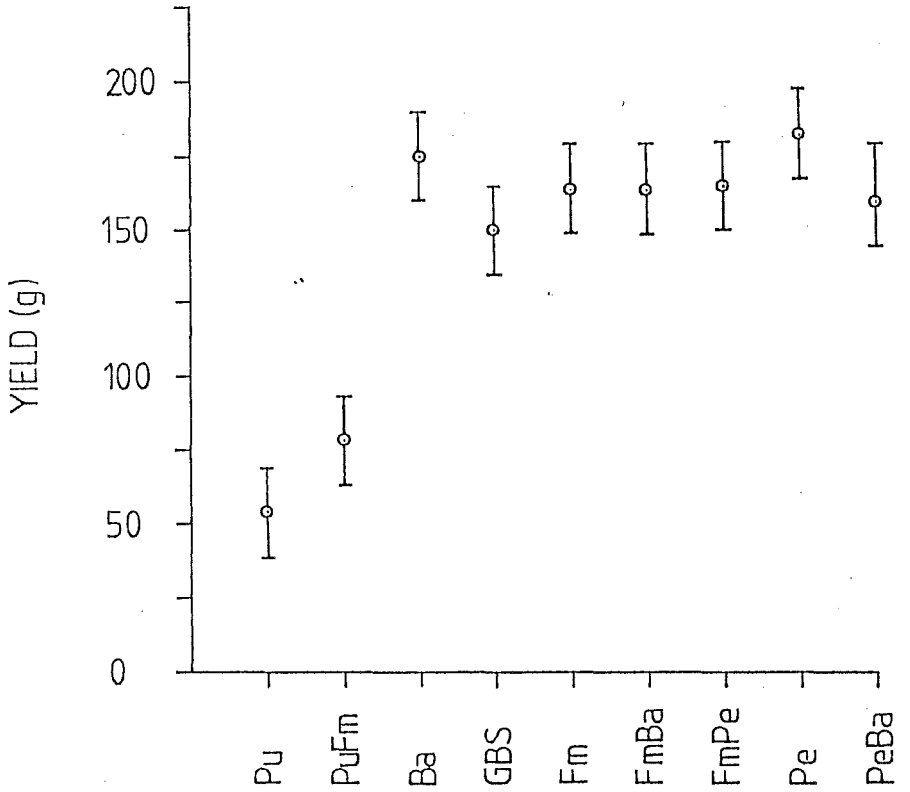
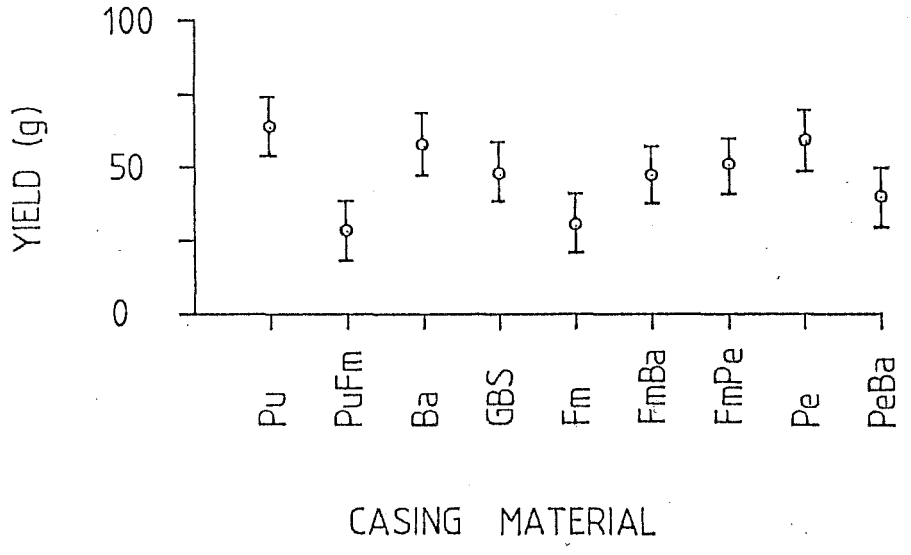


Fig 1.15. Mean yield of mushrooms from flush 3.



analysis of the 3 flushes, however, (Fig 1.12), shows the treatments do have a significant influence in terms of gross yield, despite the 'smoothing effect' of the nutrient deficient compost.

No difference was found between the water contents of the sporocarps from different casing materials. The fruiting bodies from the different casing materials contained an average of 93-94% water by weight (Table 1.4).

Mean mushroom weight is also given in Table 1.4. Discussion is deferred until section 1.3.4.

1.3.3b The occurrence of brown mould (Peziza ostracoderma)

Fm was subject to a severe out-break of brown mould in the first week after casing, ie. before the casing layer was fully colonized by the mushroom mycelium (Plates 1.1 and 1.2). The Fm mixes ie. FmPe, FmBa and PuFm were all invaded to a small extent while Ba remained unaffected. Brown mould caused a delayed first flush in Fm, the reason for the low value in Fig 1.13. The milder outbreaks of brown mould did not appear to affect the growth of the mushroom mycelium and Plate 1.3 shows the mushroom mycelium overgrowing the brown mould.

Brown mould is one of the many weed moulds that occur on mushroom beds and is often indicative of an unsuitable medium for mushroom growth (Fletcher and Atkinson 1977). Brown mould occurs on the casing layer in areas were there is no competition from other organisms and is often found on materials that have been over pasteurized. The damage caused

Table 1.4. Summary of abiotic properties of the 9 casing materials (with lime) and their effect on total yield.

CASING	Pu	PuFm	Ba	GBS	Fm	FmBa	FmPe	Pe	PeBa
PROPERTY									
CEC (me/1ØØg)	15.6	26.4	32.8	37.6	33.7	34.6	38.4	44.4	38.6
Bulk density (g/cm^3)	Ø.75	Ø.57	Ø.35	Ø.49	Ø.41	Ø.4Ø	Ø.38	Ø.38	Ø.44
Pore space (%V/V)	69	8Ø	84	77	85	85	87	88	84
Field capacity (%V/V)	49	49	4Ø	49	57	48	51	48	46
Air filled pores (%V/V) at F.C.	18	26	45	29	29	32	3Ø	34	38
First pins (days from casing)	18	11	15	17	16	16	17	15	16
First pick (days from casing)	25	22	24	25	24	24	25	24	25
Water (%W/W) content of mushrooms	92	92	93	93	93	93	93	94	93
Mean mushroom weight (g)	29.6	15.6	1Ø.5	12.2	16.8	12.1	9.6	8.3	8.5
Mean yield (Kg/Kg dry compost)	Ø.367	Ø.444	Ø.781	Ø.615	Ø.687	Ø.754	Ø.758	Ø.754	Ø.651

Plate 1.1. Peziza ostracoderma (brown mould) on Fibre-mix casing (x Ø.25).

Plate 1.2. A. bisporus mycelium (white) beginning to overgrow the brown mould (x 2Ø).

Plate 1.3. Decomposing brown mould overgrown with A. bisporus mycelium (x 2Ø).

Plate 1.1.

Plate 1.2.

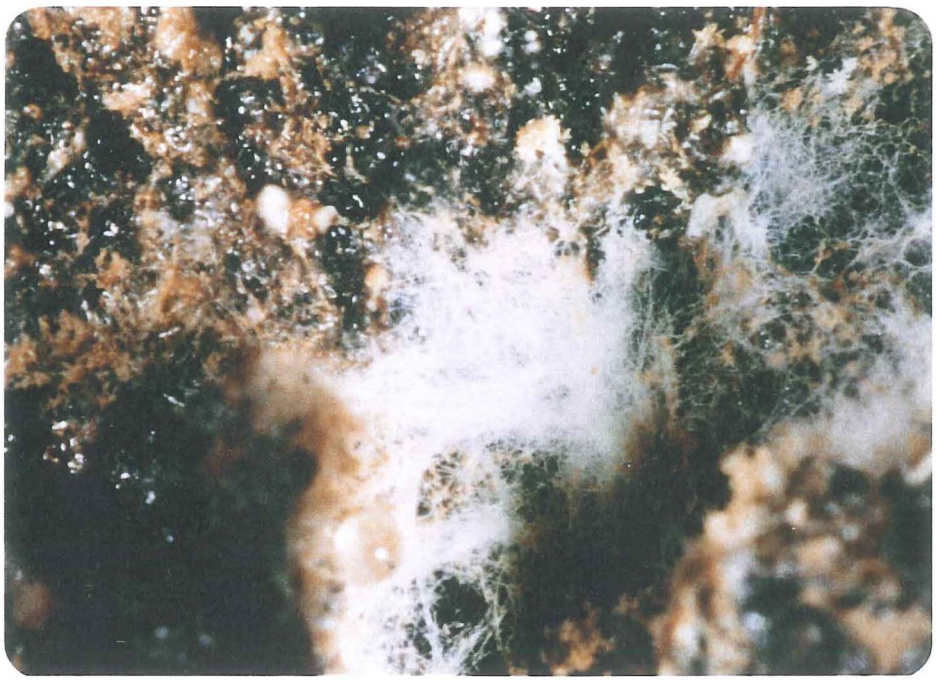


Plate 1.3.

by a severe outbreak of brown mould is usually restricted to a few days delay in picking (Vedder 1978).

The occurrence of brown mould in New Zealand is mostly confined to bark casing layers. Its absence from Ba in the cropping trial was unexpected, but may possibly be related to the length of time Ba was left to weather before use. It is possible that brown mould only occurs in the early stages of the decomposition of Ba, however, further work is required on this fungus before its effects can be properly assessed.

1.3.4 STRUCTURE OF THE CASING LAYER AND ITS EFFECT ON YIELD AND MEAN MUSHROOM WEIGHT

1.3.4a Bulk density and porosity

Examination of Table 1.5 reveals the marked effect of structure on both yield and mean mushroom weight. A highly significant, inversely proportional relationship between bulk density and yield indicates the unsuitability of dense media to function as productive casing materials. Bulk density also appears to influence the size of the sporocarps; the more dense the media, the larger the mushrooms.

Examination of the correlation and regression statistics relating to porosity (Table 1.5) shows that materials with large pore volumes, such as Ba or Pe, tend to produce large yields (Table 1.4). This result is not unexpected considering the close relationship between bulk density and porosity. An inverse relationship exists between porosity and mean mushroom weight.

Table 1.5. Correlation and regression statistics showing the linear relationship between parameters of structure and both yield and mean mushroom weight for 9 treatments.

Parameters of structure	Statistic	Yield	Mean mushroom weight
Bulk density	Pearson's	r = -Ø.97	r = +Ø.88
	F stat	***	**
	Slope	***	**
Porosity	Pearson's	r = +Ø.87	r = -Ø.84
	F stat	**	**
	Slope	**	**
Air filled pores	Pearson's	r = +Ø.76	r = -Ø.79
	F stat	**	**
	Slope	*	*
Water content	Pearson's	r = -Ø.12	r = +Ø.29
	F stat	NS	NS
	Slope	NS	NS

Pearson's = Pearson's correlation coefficient (r)

F stat = Significance of linear fit

Slope = Significance of the slope of the regression line (H_o: B = Ø)

NS = Not Significant

* = P < Ø.Ø5, ** = P < Ø.Ø1, *** = P < Ø.ØØ1

1.3.4b Air-filled pores and water content

The correlation and regression statistics presented in Table 1.5 reveal a significant relationship between the volume of air-filled pores and yield. Ba, which had 45% (V/V) air-filled pores, produced yields considerably greater than Pu and PuFm which had 18% (V/V) and 26% (V/V) air-filled pores respectively. Mean mushroom weight is inversely correlated with air-filled pores, thus casing materials containing a small volume of air tend to produce large mushrooms.

Within the range of water contents examined, the amount of water contained within the pores of the casing materials at field capacity appears to have no influence on yield or mean mushroom weight. This result contrasts with the traditional views expressed by Flegg (1954), Reeve et al. (1959), Visscher (1982) and others who report that a productive casing material must have a large water holding ability. The example of Ba, which had the lowest water holding ability of the casing materials examined in the cropping trial (40% (V/V) at field capacity) strongly indicates that to be productive a casing material need not have a large water retaining capacity. It is unlikely that the crop from Ba was in any way water-stressed or that the yield could have been increased by raising the water content of the casing material. Had this been the case, the average water content of the sporocarps from Ba, would have most likely been lower than the average water content of those from other casing materials (see Table 1.4).

In a commercial situation, the casing layer is watered at least twice a day except during pin formation (a period of 2-3 days) when watering is reduced or stopped (Vedder 1978). Thus the ability of materials with low water holding capacities to function as productive casing materials is not surprising. There is undoubtedly a minimum water holding capacity below which productivity decreases. Reeve et al. (1959) demonstrated that a casing soil with a water content of 3Ø% of field capacity will not yield as well as a casing soil with a water content of greater than, or equal to 6Ø% of field capacity. Unfortunately they presented their results on a weight basis without giving an indication of bulk density, thus meaningful comparison of results is not possible.

A number of investigations have been made into the importance of air in the casing layer. Several of these support the findings of this study (Bels Koning 195Ø, Edwards and Flegg 1954, Tschierpe 1972, Hayes and Nair 1974, Nair et al. 1976). The most recent examination of the effect of different levels of air and water in the casing layer on yield was by Hayes (1981). The conclusions reached, however, that air and water levels in the casing layer are not critical factors influencing productivity, are open to criticism on the following grounds:

(1) The formula which Hayes used for calculation of 'pore space %' leads to confusion as to whether he measured air-filled pores or air-filled pores plus water-filled pores.

(2) 'Water holding capacity' (which is incorrectly calculated) is expressed on a weight basis and therefore the

comparison with 'pore space %' (expressed on a volume basis) is meaningless (pers. comm. J.B. Reid, Soil Science Dept., Lincoln College, Lincoln. L.G. Greenfield, Dept. of Plant and Microbial Sciences, University of Canterbury, Christchurch).

It is quite possible that recalculation of the results presented by Hayes may lead to a different conclusion regarding the importance of air in the casing layer.

Visscher's ideas on the value of a compact casing layer, (1975, 1979), appear to conflict with the views of other workers (as discussed previously). It seems, however, that both views are equally valid, the balance between ideas being dependent on the structure of the casing material. This point is highlighted by the performance of Pu in the cropping trial. According to Visscher's theory, the most dense medium, Pu, if ruffled immediately prior to fructification, should have produced the largest yield. This is not the case suggesting factors other than high CO_2 levels are important in influencing sporocarp production. Analysis of the physical and chemical properties of Pu suggest that yield could have been improved by following the recommendations of Hayes and Nair (1974) and others who recommend an open structured casing material, (the results presented in section 1.3.2a also indicate this). Alternatively, a very open structured material such as a spongy peat augmented with lime chips (it is possible that the material used by Visscher was of this nature) could be made more productive by compacting, resulting in increased vegetative mycelial growth brought

about by higher CO_2 levels within the casing layer (visscher 1975, 1979).

Visscher does not appear to take into account the extremely complex interactions between the physical, chemical and biological properties of the casing layer environment, consequently the correctness of the theory he presents to explain the increased yields from compacted, ruffled casing materials is questionable. A more precise interpretation of his results should consider the following:

(1) The effect of pH on the solubility of CO_2.

Nair et al. (1976), reported that when a unit volume of CO_2 is dissolved in water at pH 7.5 - 8.Ø, approximately 95% will be present in the aqueous phase as the bicarbonate ion and 5% in the gaseous phase. In a wet environment such as the casing layer, the mycelium will be bathed in a film of water (Boddy 1984). Macauley and Griffin (1969) demonstrated that when this occurs, the bicarbonate ion and not CO_2 is most likely responsible for effecting the growth of the mushroom mycelium.

(2) The importance of O_2.

Most fungi require O_2 for respiration and oxidative assimilation (Hayes and Nair 1974). The mushroom mycelium depends on the gaseous phase (which is in turn dependent on the existence of air-filled pores) for its supply of O_2, because unlike CO_2, O_2 is relatively insoluble in water (Boddy 1984). This fact highlights the importance of air-filled pores in the casing layer. The volume of O_2 present in the casing layer is likely to be small compared to the amount

of bicarbonate, (especially if the pore volume is small), however, as many soil fungi are relatively insensitive to O_2 levels, mycelial growth at low O_2 concentrations presents few problems (Griffin 1968).

(3) Bacterial interactions.

The many beneficial bacteria in the casing layer which are species of Pseudomonas, are aerobic (Stanier et al. 1966). Hayes and Nair (1974) found that better aeration of the casing layer through the use of a more open structured material led to an increase in the numbers of beneficial bacteria, which in turn increased the yield of mushrooms. Visscher (1975, 1979) also acknowledges the importance of bacteria, however, argues that a more open structured casing material means the loss of volatile substances produced by the mushroom mycelium. These substances, ethanol, acetone etc., have been shown to stimulate the growth of Pseudomonas spp. (Lockard and Kneebone 1962, Hayes and Nair 1976). These volatile substances are continuously produced by the mushroom mycelium, therefore it is unlikely that a more open structured casing material would cause a decrease in the activity of the pseudomonads. If the action of ruffling the casing layer is also taken into account then Visscher's explanation for the increased yield, following ruffling, seems inadequate. Ruffling the casing layer would most likely lower the CO_2 levels in the casing for only a short period and amounts of the more important bicarbonate ion would probably not be affected. The first watering following

ruffling would also presumably quickly fill many of the pores that had been created by ruffling, thereby reverting the casing later to its previous compact state. Furthermore, as long as the air above the casing layer is inducive to fructification, ie. has a low CO_2 concentration, then it actually seems advantageous to maintain bicarbonate (CO_2) levels in the casing which continue to encourage vegetative mycelial growth. Thus the increased yield following ruffling is more likely a result of the improved colonization of the casing layer rather than lowered CO_2 levels.

It would be desirable to repeat Visscher's experiment in order to assess the effect of compaction on the casing materials, especially Pe and Ba, used in the cropping trial.

Structure appears to have a significant effect on mean mushroom weight (Table 1.5) although the reason for this is unknown. The theory proffered in section 1.3.2b, to explain the relationship between dense casing materials and hyphal mass, also provides a possible explanation for this phenomenon. Compacting the spongy casing materials, such as Pe, FmPe and PeBa, would most likely have increased the weight of the sporocarps produced by these materials.

1.3.4c Lime

The effect of lime on the physical properties of the casing materials is difficult to assess. It is possible that lime has a beneficial effect on open structured materials but a detrimental effect on dense casing materials. It should be stressed that the lime used in the cropping trial was Oxford

chalk-lime, fine textured and fairly soluble and that the trends shown in this trial will not be applicable to all types of chalk-lime. There is definite need for a thorough examination of the role of lime in the casing layer. Two aspects, in particular, require investigation:

(1) Rates of application. The results of Hayes (1981) suggest that small volumes of lime, as little as 2%, are more favourable than large volumes. The relevence of this result to New Zealand conditions needs investigation.

(2) The effect of different chalk-limes on yield, particularily the effect of a fine textured lime compared to a coarse textured lime.

1.3.5 CHEMICAL PROPERTIES OF THE CASING LAYER AND THEIR EFFECT ON YIELD AND MEAN MUSHROOM WEIGHT

1.3.5a Electrical conductivity

Electrical conductivity of the casing layer at day Ø, (the day of casing), not unexpectedly, shows no relationship with yield or mean mushroom weight. Electrial conductivity at the end of the cropping trial (day 48) although still not statistically significant does show a negative relationship with yield and a positive relationship with mean mushroom weight (Table 1.6).

The conclusions reached by Yeo and Hayes (1979) and Hayes (1981) concerning the influence of soluble salt levels on yield are questionable. More direct evidence of the effects of soluble salts on the growth and fruiting of A. bisporus is required before placing too much emphasis on

Table 1.6. Correlation and regression statistics showing the linear relationship between 2 chemical properties of the 9 casing materials and both yield and mean mushroom weight.

Chemical properties	Statistic	Yield	Mean mushroom weight
Electrical conductivity (day Ø)	Pearson's	r = +Ø.Ø6	r = +Ø.15
	F stat	NS	NS
	Slope	NS	NS
Electrical conductivity (day 48)	Pearson's	r = -Ø.54	r = +Ø.55
	F stat	NS	NS
	Slope	NS	NS
Cation exchange capacity	Pearson's	r = +Ø.82	r = -Ø.92
	F stat	**	***
	Slope	**	***

Pearson's = Pearson's correlation coefficient (r)

F stat = Significance of linear fit

Slope = Significance of the slope of the regression line ($H_{Ø}$: B = Ø)

NS = Not significant

* = P < Ø.Ø5, ** = P < Ø.Ø1, *** = P < Ø.ØØ1

the correlation between increasing levels of soluble salts over time and decreasing yield. The depletion of nutrients by A. bisporus (and not the increasing levels of soluble salts) appears the most obvious explanation for the decrease in yield over time.

1.3.5b Cation exchange capacity

The significant correlation and regression between both yield and mean mushroom weight suggests that the available nutrient ions in the casing layer affect the growth and development of A. bisporus. It is interesting to note that CEC has a positive effect on yield, while soluble salts have a negative influence. This suggests not all ions in the casing layer have a beneficial effect on the growth of A. bisporus. As a resut of the complex interactions occurring in the casing layer and considering the general nature of this experiment, it would be inappropriate to speculate further on the role of exchangeable cations in the casing layer. Further work is required to investigate in more detail the importance of the casing layer as a source of nutrients for both A. bisporus and the general microbial flora.

1.3.6 GENERAL OBSERVATIONS AND COMMENTS

1.3.6a Ease of picking.

The sporocarps from Fm came away cleanly from the surface of the bed and no casing remained attached to the mature sporocarps. Ba and GBS both produced clean mushrooms, however, they were not as easily removed as were those from

Fm. Pe often produced 'dirty' mushrooms suggesting there was a tendency for pins to form slightly below the surface with this material, possibly a result of the spongy nature of Pe. Mushrooms from Pu were difficult to remove and usually had to be cut from the casing layer to prevent large sections of casing being removed with each sporocarp.

1.3.6b Over-pinning

Pe tended to 'over-pin', producing a mass of mushroom initials, many of which failed to fully develop. A similar effect was observed with PeBa and FmPe. This maybe related to the spongy nature of Pe. Further work on the effect of compaction in attempting to control this problem is indicated.

1.3.6c Deformed sporocarps

Pu often produced sporocarps with malformed caps, most likely a result of damage inflicted on the young mushroom initials by sharp pumice fragments (pers. comm. F.R. Sanderson, DSIR. Lincoln.). Deformed mushrooms were also found on PuFm, few were found on other casing materials.

1.3.6d Novel casing materials.

A number of spawn-run compost cubes were buried on the Port Hills, Canterbury; 3 cubes were cased with pine litter and 3 with Hoheria leaf litter. Both 'natural' casing materials produced a crop of mushrooms.

In the second mushroom growth cabinet at Lincoln, 3 boxes of spawn-run compost were cased with 4 sheets of

newspaper and 3 were left uncased. The newspaper treated boxes each produced 2-3 large, (> 100g), mushrooms, while the uncased boxes failed to yield any sporocarps again highlighting the role of the casing layer.

1.4 CONCLUSION

Nine different casing media were examined for their ability to promote mushroom production. Ba, FmBa, FmPe and Pe proved to be the most productive followed closely by GBS, Fm and PeBa. Pu and PuFm were considerably less effective.

Analysis of the physical properties of these materials revealed that the structure of the casing layer was an important factor affecting the fruiting of A. bisporus. Air-filled pore volume appears a significant factor, while the volume of water-filled pores seems to bear no relation to yield. Lime has an important role, its influence appears beneficial when added to open structured materials, but detrimental when added to dense materials. The use of different lime types, thus effecting air-filled pore volume, may provide a low cost and useful way of manipulating the structure of the casing layer. This, however, requires further investigation.

Chemical analysis of the casing materials indicated that the nutrient status of the casing layer, as reflected by cation exchange capacity, has a positive effect on yield. The previously reported negative effect of soluble salts levels on yield was not confirmed. Lime appears to have a beneficial effect on the chemical properties of the casing layer, however, the extent of its effect depends on the nature of the material with which it is mixed.

1.4.1 EVALUATION OF CASING MATERIALS

1.4.1a Bark

Of the 9 casing materials, Ba was the most productive. GBS was not as effective and this maybe related to the small air-filled pore volume of GBS and a detrimentally high phenolic content, a result of insufficient weathering before use.

1.4.1b Fibre-mix

Fm did not yield as well as expected, a result of two factors; the outbreak of brown mould and the small volume of air-filled pores. It is possible that Fm, if mixed with a coarse, relatively insoluble lime, (to increase air-filled pore volume), could be made as productive as either Pe or Ba. Failing this, the low cost of Fm ($5.ØØ per m^3, compared with $18.ØØ per m^3 for Ba and approximately $45.ØØ per m^3 for Pe) coupled with the apparent ability of the material to mix with Pe and Ba without effect, indicates the value of Fm as a low cost matrix, or 'filling' agent.

1.4.1c Peat

Pe produced yields equal to the 3 other high yielding casing materials in the cropping trial. The average sporocarp weight from Pe was low.

1.4.1d Pumice

Pu was the poorest yielding casing material - it had little air-filled pore space. The sporocarps from Pu were large, often deformed and difficult to harvest. Despite these

problems, Pu is currently used by one North Island grower suggesting that under certain conditions of management it can produce satisfactory yields. Communication with this grower revealed that he adds less of a coarser structured lime which presumably increases the volume of air-filled pores to a satisfactory level. The addition of Fm to Pu only slightly improved its performance.

2.Ø INVESTIGATIONS INTO THE ROLE OF BACTERIA IN SPOROPHORE INITIATION

2.1 INTRODUCTION

Sporophore initiation in A. bisporus, despite much investigation, remains incompletely understood (Long and Jacobs 1974). Research attempting to elucidate factors responsible for the transition from vegetative to reproductive growth has focused on two main areas (1) The involvement of CO_2 and volatile metabolic compounds. (2) The involvement of soil microbes.

2.1.1 THE INVOLVEMENT OF CO_2 AND VOLATILE ORGANIC COMPOUNDS

2.1.1a CO_2

The damaging effect of high levels of CO_2 on sporophores was demonstrated by Lambert (1933). In 1959 Tschierpe extended Lambert's work and reported that a CO_2 concentration of Ø.5% in the air above the casing layer inhibited mushroom initiation. He postulated that the CO_2 gradient between the compost (high CO_2) and the air of the mushroom house (low CO_2) was responsible for sporophore initiation. This hypothesis was disputed by Thomas et al. (1964) who were able to initiate sporophores in petri-dishes even after destroying the CO_2 gradient by physically separating the casing from the compost. Further evidence for the involvement of CO_2 in the control of sporophore initiation was presented by Tschierpe

and Sinden (1964). They found that for some mushroom strains, CO_2 concentrations of between Ø.ØØ3% and Ø.12% in the air above the casing stimulated maximum initiation of sporocarps. They also demonstrated that mycelial strands, when exposed to low concentrations of CO_2 for 24h, formed mushroom initials. Long and Jacobs (1968) examined the response of vegetative hyphae to CO_2 in both sterile and non sterile casing soil. They found that under axenic conditions hyphal growth was proportional to the CO_2 concentration over the range 22-37Ø ppm. At high concentrations of CO_2 there was no increase in the growth rate. Under non-axenic conditions they discovered that hyphal growth was proportional to the CO_2 concentration over the range Ø-1Ø4 ppm, while between 1Ø4 and 1ØØØ ppm CO_2 strand growth was inhibited and sporophore initials were formed. Thus CO_2, although being important in the process of sporophore initiation, was shown not to be the controlling factor.

2.1.1b Organic substances

The involvement of volatile substances in sporophore initiation was first implied by Mader (1943) who suggested that unidentified volatiles exist, which unless removed by ventilation inhibit sporophore development. Stoller (1952) also postulated the existence of a volatile substance other than CO_2. He suggested that the substance was inhibitory to fructification and that the casing layer, being an alkaline-oxygenated medium, was responsible for its destruction.

Five volatile substances; acetone, ethylene oxide, ethyl

actetate, acetaldehyde and ethanol were shown to be produced by A. bisporus (Lockard and Kneebone 1962). Ethanol, however, is most likely a result of anaerobic metabolism by A. bisporus (Tschierpe and Sinden 1965) and as fruiting of the mushroom ceases under conditions of reduced ventilation it is unlikely that ethanol is a crucial factor in the process of sporophore initiation (Eger 1972). Turner et al. (1975) also examined volatile substances produced by A. bisporus in the compost. They found nine volatile hydrocarbons of which only ethylene showed any correlation with crop development; high levels of ethylene coincided with rapid enlargement of fruiting bodies. Wood and Hammond (1977) and Ward et al. (1977) examined further the role of ethylene and demonstrated that its production was restricted to mycelium and concluded that it had no regulatory role in the growth and fruiting of A. bisporus.

Visscher (1979) produced evidence indicating that ethylene interacted with CO_2 stimulating sporophore initiation and production. He suggested this phenomenon was similar to the ethylene/CO_2 antagonism in plants and demonstrated that air containing ethylene at a concentration of Ø.ØØØ1% and CO_2 at a concentration of Ø.Ø3%, provided conditions most favourable for sporophore initiation. Visscher used unsterile casing soil in his experiments and while acknowledging the importance of soil microbes was unable to explain their possible role with respect to his theory of ethylene/CO_2 antagonism.

2.1.2 INVOLVEMENT OF SOIL MICROBES

The 'Halbschalentest', a laboratory method for assessing the effects of different casing materials on sporophore initiation, was developed by Eger (1961). She discovered that sporophore initiation was suppressed when autoclaved casing materials were used thus indicating the importance of microbes in this process. Eger also found that activated charcoal could replace the effects of micro-organisms and stimulate fructification. This was later confirmed by Long and Jacobs (1974), Couvy (1976) and Peerally (1979). On the basis of these results Eger suggested that the microbes (bacteria) controlled sporophore initiation by removing metabolites released from the hyphal tips of A. bisporus. Eger (1962) reported that mycelial growth was inhibited by the bacteria and that fruiting was associated with this check in growth.

O'Donoghue (1962) observed sporophore production in what was thought to be a pure culture of mushroom spawn. Closer examination of the spawn revealed the presence of actinomycetes which in the light of Eger's (1961) findings were presumed responsible for sporophore initiation.

Urayama (1967) reported the isolation of a metabolite from B. psilocybe which induced sporophore formation in a range of agarics. This isolate, however, has since become ineffective (as quoted by Hayes 1969). Urayama also noted that B. psilocybe was responsible for stimulating both mycelial growth and sporophore formation.

Hayes *et al*. (1969) confirmed and extended the work of Eger (1961, 1962). They used known volatile metabolites of *A. bisporus* to select bacterial populations which were able to stimulate sporophore initiation. Further examination of these stimulatory bacteria revealed them to be related to *Pseudomonas putida*. Yeasts and micro-algae were also shown to increase sporophore production and mycelial density (Curto and Favelli 1972). Park and Agnihotri (1969a, 1969b) claimed that a range of soil bacteria and their culture filtrates would promote fruit body initiation. They also reported the stimulatory effects of chemicals such as biotin and oxalic acid on sporophore initiation, however, Eger (1972) and Wood (1976) were unable to confirm their results.

An in depth study of sporophore initiation in relation to bacteria and charcoal was conducted by Peerally (1979) who was able to induce localized 'pin' formation by locally inoculating pure cultures of *A. bisporus* with a mixed bacterial suspension. This result led him to suggest that close contact between bacteria and mycelium is crucial in the control of fruiting and that volatile substances (other than CO_2) may not be as important as previously thought. He also described the occurrence of mycelial strands following the addition of either activated charcoal or a mixed bacterial suspension to a pure mushroom culture and concluded his study by suggesting that bacteria modify the pattern of mycelial growth, encouraging strand formation, which finally leads to fruiting.

The production of primordia *in vitro* is not a true

reflection of the situation within the casing layer, nevertheless, meaningful study of sporophore initiation requires that sporophore initials be produced in a controlled environment. Several authors have developed systems for this purpose. Hume and Hayes (1972) devised two petri-dish techniques using 2% malt extract agar as a nutrient source (one for 'fluffy' strains of A. bisporus and the other for 'strandy' strains). Both techniques rely on the close association between A. bisporus and P. putida for the production of mushroom initials. Hume and Hayes reported that controls without bacteria rarely produced primordia. Other techniques allowing study of the physiology of sporophore initiation have involved the use of cased grain spawn (San Antonio 1971), petri-dish methods using soil (Eger 1961, Peerally 1979) and a modification of the Hume and Hayes (1972) method by (Peerally 1981).

Hayes (1972) using the petri-dish method of Hume and Hayes (1972) as a means of investigating nutritional factors in relation to sporophore initiation found that iron containing compounds and chelating agents, when added to axenically grown plate cultures of A. bisporus promoted primordium formation. This discovery led Hayes to suggest that bacteria, in particular P. putida, control the process of sporophore initiation by releasing iron from the organic chelating agents in the casing layer. Neilands (1974) demonstrated the ability of P. putida strains to produce iron-binding compounds such as sideramines and siderchromes.

Wood (1976) investigated the theory of Hayes (1972) taking into account the results of Neilands (1974). He found no evidence, however, to suggest that strains of P. putida stimulate primordium formation through the production of iron-binding compounds. In 1981, Hayes again examined the role of bacteria in the casing layer with respect to iron and sporophore initiation and his results tended to confirm his earlier (1972, 1974) work. Hayes demonstrated that under axenic conditions, levels of water soluble iron increased with time, while under non-axenic conditions levels remained at a constant low level. He suggested that water soluble iron (produced by the mushroom mycelium and possibly inhibitory to its own growth at some concentration level (Hayes 1972)) was fixed by bacteria into an insoluble form, thus maintaining levels of water soluble iron at concentrations favourable for the growth and fruiting of A. bisporus.

2.1.3 BACTERIAL ECOLOGY OF THE CASING LAYER

Examination of the gross ecology of the casing layer has provided further valuable evidence indicating the importance of bacteria, particularily species of Pseudomonas, in sporophore initiation (Hayes and Nair 1976). The dominance of Pseudomonas species in a peat based casing material 1Ø days after casing was shown by Hayes and Nair. They were also able to demonstrate, in pure culture, an increase in the numbers of a Pseudomonas isolate by enriching the atmosphere in which it was growing with volatile compounds emitted from mushroom mycelium growing on compost, thus indicating that the

mushroom mycelium is responsible for selecting and maintaining its own bacterial flora.

Cresswell and Hayes (1979) detected 2 peaks of bacterial activity in the casing layer, the first coinciding with fruitbody initiation and the second at the time of the third break. They confirmed Hayes and Nair's (1976) results regarding the dominance of Pseudomonas, especially P. putida, but also found bacteria of the family Enterobacteriaceae and the genus Alcaligenes to be common inhabitants of the casing layer. The importance of the compost as a potential source of bacterial inoculum for the casing layer was also shown by these authors.

Pseudomonads are a widespread and diverse group of bacteria with many unique characteristics. These include the ability to cause disease in both plants and animals, degrade many materials, including highly recalcitrant compounds and antibiotic production. Many of these abilities have been shown to be controlled by the presence of extrachromosomal genetic elements (plasmids) (Chakrabarty 1976, Timmis and Puhler 1979). It was considered possible that the special characteristics of P. putida responsible for triggering sporophore initiation in A. bisporus may be plasmid borne.

2.1.4 PLASMIDS IN Pseudomonas

Plasmids have been reported in several species of the fluorescent pseudomonads. R factor (for the inactivation of potent antibiotics) and mercury resistance plasmids have been

discovered in P. aeruginosa. Sex factor plasmids which are capable of initiating chromosomal gene transfer from one cell to another are known for both P. aeruginosa and P. putida. Degradative plasmids such as CAM, TOL, SAL and OCT have been studied in P. putida (Chakrabarty 1976). The presence of one or more plasmids in P. phaseolicola has also been shown (Panopoulos et al. 1979).

Nearly all the plasmids studied in the fluorescent Pseudomonas group are between 77-140 kb (Martin 1982). Hansen and Olsen (1978) reported that some are as large as 480 kb and suggested that many genes whose functions are as yet unknown are also carried on these plasmids.

Investigations aimed at the identification, isolation and manipulation of plasmids are considerably aided by the presence of marker genes, ie., plasmid borne genes which encode for factors such as antibiotic and heavy metal resistance. These marker genes allow rapid and certain identification of the presence of plasmid(s) (pers. comm. H.K. Mahanty, Dept. Plant and Microbial Sciences, University of Canterbury, Christchurch.). Mercury resistance is nearly always plasmid borne and often associated with antibiotic resistance as is the case with P. aeruginosa (Chakrabarty 1976). It was considered possible that genes encoding for mercury resistance may also be present in sporophore inducing strains of P. putida and would act as a suitable marker if present.

2.1.5 STUDY AIMS

This study endeavoured to isolate and characterize strains of *P. putida* from a range of different casing materials, water and soil. The ability of a single *P. putida* isolate to stimulate the initiation of mushroom sporophores was examined. A reliable assay system allowing the production of primordia *in vitro* is required in order to provide a means of studying the interaction between *A. bisporus* and *P. putida*. For this purpose the *in vitro* techniques reported in the literature were investigated, assessed and modified. Physiological and genetical experiments were conducted in an attempt to elucidate the role of *P. putida* in the process of sporophore initiation.

2.2 MATERIALS AND METHODS

2.2.1 ISOLATION OF BACTERIA

It is possible to enrich for fluorescent pseudomonads by taking advantage of their ability to use nitrate as a source of cellular nitrogen and also their ability to use the salts of various organic acids as carbon and energy sources (Stanier et al. 1966).

A sample, Ø.1 g, of either fresh casing material, soil or river or pond water (where a Ø.1 ml sample was taken) was added to 3 ml of succinate salts medium (Krieg 1981) and incubated for 24 h at 3Ø C. A secondary enrichment culture was prepared before streaking onto solidified succinate salts medium. Following a further 24-48 h incubation period individual colonies were picked from the succinate salts medium with sterile toothpicks and transferred to plates of King's medium B (KB) (King et al. 1954). After growth of the colonies (24 h) the plates were examined under ultraviolet light (254 nm) and fluorescent colonies selected for further characterization. Mueller Hinton medium (Difco) was also used as means of detecting fluorescent bacteria, allowing identification of these isolates after only 8 h. Purity of isolates was checked by restreaking onto KB.

2.2.2 MORPHOLOGICAL AND BIOCHEMICAL TESTS

2.2.2a Colony morphology

Colony morphology was recorded from KB plates after 48 h

growth.

2.2.2b Gram-stain

Huckers method for Gram-staining was employed.

2.2.2c Oxidase

Kovacs' (1956) test was used with Ø.1% ascorbic acid added to the 1% tetramethyl-p-phenylenediamine dihydrochloride solution to retard autoxidation. The test was conducted on filter paper using young (< 24 h) cultures. Positive and negative test organisms, P. aeruginosa and Escherichia coli respectively, were used as controls.

2.2.2d O-F test

The medium of Hugh and Leifson (1953) was used with glucose as the carbohydrate source. Tubes without glucose were set up in parallel to allow more precise determination of the indicator changes due to assimilation of the carbohydrate (Hendrie and Shewan 1979). Tubes were incubated at 3Ø C for 2 weeks.

2.2.2e Hydrogen-sulphide production (TSI)

Deep butted slopes of triple sugar iron (TSI) (Anon. 1958) agar were stab and streak inoculated then incubated at 3Ø C for 24 h. A blackened butt indicated production of H_2S. TSI agar also provided information regarding the ability of the isolate to ferment the sugars glucose, lactose and sucrose. Proteus vulgaris was employed as a positive control.

2.2.2f Motility

Motility was determined by examination of 12 h cultures by the 'hanging-drop' method.

2.2.2g Flagella

Presence of flagella was determined using Ryu's stain following the method of Kodaka et al. (1982). KB was used instead of blood agar.

2.2.2h Pigment production

King's medium A (KA) (King et al. 1954) was used for the detection of fluorescent green-blue phenazine pigments (pyocyanine) and KB for the detection of yellow-green fluorescent pigments. Non-fluorescent pigments such as phenazine-1-carboxylate were also detectable on KB (King et al. 1954). Plates were streaked and incubated at 30 C. Examination for fluorescence was made after 24 h using an ultraviolet lamp (254 nm). A fluorescent zone in the agar surrounding the bacterial colony was regarded as positive.

2.2.2i Growth at 41 C and 4 C

Isolates were streaked onto KB plates in pairs. Plates incubated at 41 C were examined for growth after 48 h while those at 4 C were observed after 7 days. Isolates were subcultured and the procedure triplicated before estimating growth (Haynes and Rhodes 1962).

2.2.2j Gelatin hydrolysis

MacCartney bottles of nutrient gelatin were stab inoculated and incubated at 25 C for 7 days. Before recording

results the cultures were placed at (4 C) for 3Ø min. Very fluid cultures were considered positive, while viscid types were considered negative (Zarkower et al. 1984). Sterile distilled water was used as a control.

2.2.2k Arginine dihydrolase

The method of Thornley (196Ø) was used, with sterile distilled water as a control.

2.2.21 Starch hydrolysis

Isolates were streaked onto plates of nutrient agar + Ø.8% starch and after 48 h incubation at 3Ø C the plates were treated with Gram's iodine (Hendrie and Shewan 1979).

2.2.2m Levan production

Levan production was detected by the growth of domed mucoid colonies on nutrient agar + 5% sucrose. The plates were examined after 48 h incubation at 3Ø C (Lelliott et al. 1966). Nutrient agar (no sucrose) was inoculated as a control.

2.2.2n Nitrate reduction

The method of Zarkower et al. (1984) was followed however a semi-solid medium was used (Appendix 3). Bijou bottles were half filled with medium, inoculated and incubated at 25 C. After 7 days reagents A and B (Appendix 3) were added to each bijou bottle. Development of a cherry-red colour indicated presence of NO_2. If no colour change occurred within 3Ø min, Zn dust was added. Absence of a red

colour after after the addition of Zn dust indicated complete denitrification. P. aeruginosa was employed as a positive control organism.

2.2.3 CULTURE OF BACTERIA

Bacteria were grown in shake cultures (150 ml) of nutrient broth (BBL) at 25 C and harvested at the end of the exponential phase of growth. The cells were separated from the medium by aseptic centrifugation and washing (Hume and Hayes 1972, Fermor and Wood 1981).

2.2.4 PREPARATION OF WATER AGAR PLUGS CONTAINING BACTERIA

Bacteria were cultured as in 2.2.3 and suspended in sterile distilled water at a concentration of 1×10^8 cells/ml. One ml of this suspension was then added to 19 ml of autoclaved water-agar (1.5% agar) immediately prior to solidification. Once solid, discs were removed using a No. 2 cork borer (5.0 mm diameter) (Hume and Hayes 1972).

2.2.5 MUSHROOM CULTURES

A number of strains of A. bisporus (grain culture) were obtained; strains 208, L1, B8, Ax60 and B92 were kindly supplied by the Mushroom Spawn Laboratory (Waikato) Ltd New Zealand and strains 649, ML3, and ML4 were kindly donated by Meadow Mushrooms, Prebbleton, New Zealand. Petri-dish cultures of each strain were prepared by aseptically

transferring the mycelium from the surface of the grain spawn to 2% malt extract (Difco) agar. After 14 days incubation at 25 C sub-cultures were prepared. Original stock cultures were maintained om malt extract medium at 4 C (Hume and Hayes 1972).

2.2.6 MEDIA FOR THE CULTURE OF A. bisporus

Malt extract (2% malt extract, 1.5% agar, pH 7.5) is the preferred nutrient medium for in vitro cultivation of A. bisporus (Hayes 1972, Smith and Hayes 1972, Hume and Hayes 1972, Fermor and Wood 1981) and was used in this study. Preliminary experiments, however, revealed that malt extract (Difco) while encouraging more vigorous growth than potato dextrose agar (Difco), was not able to support extended vigorous growth of A. bisporus. Fermor and Wood (1981,1982), Sparling et al. (1982) and Wood and Fermor (1982) suggested that the microbial biomass in the compost was an important nutrient source for A. bisporus. Fermor and Wood (1981) demonstrated the ability of A. bisporus to degrade dead microbes and reported growth of A. bisporus on Treschow basal salts medium with B. subtilis cells as the sole carbon and nitrogen source. These results provided a basis for the development of the following medium. Bacteria, B. subtilis and P. putida, were grown in shake cultures (2.2.3) and the bacteria harvested from each 150 ml flask were added to 100 ml of malt extract agar (pH 7.5) and autoclaved (121 C, 20 min). The molten medium was poured into standard volume

(9 cm diameter) petri-dishes and stored at 4 C.

An experiment was conducted in order to compare the growth of A. bisporus (strain B92) on malt extract plus B. subtilis, malt extract plus P. putida and malt extract media. Each treatment was replicated 5 times and the cultures incubated at 25 C, colony diameter was measured every 2-3 days. Observations on growth characteristics, such as the tendency to form strands, the nature of the aerial mycelium etc., were also made.

2.2.7 SPAWN PREPARATION

Spawn was made following the method of Stoller (1962), 1 l flasks were quarter filled with the prepared wheat grain and autoclaved for 1 h (15 PSI) on 3 successive days before inoculation.

2.2.8 CASING MATERIAL PREPARATION

A commercial peat casing material (25% lime, pH 7.6) was used. Approximately 250 ml of the medium was dispensed into 1 l flasks, moistened with distilled water and autoclaved for 1 h (121 C) on 3 successive days. Sterility was checked by streaking onto nutrient agar plates.

2.2.9 IN VITRO FRUITING OF A. bisporus

2.2.9a The methods of Hume and Hayes (1972)

(1) The single phase method for 'fluffy' strains

was attempted. Standard 9 cm petri-dishes containing 25 ml malt extract medium were centrally inoculated with one of 3 strains of A. bisporus, ML4, Ax6Ø or B92 and incubated at 25 C for 14 days at a relative humidity of between 5Ø and 7Ø%. A number of water agar discs (4-5), containing one of 6 fluorescent pseudomonads isolated from casing materials, were then placed at the edge of each colony and the petri-dishes incubated again at 25 C at a relative humidity of between 4Ø and 5Ø%. Relative humidity was controlled by placing beakers of water with differing surface areas inside the incubator. Each treatment was triplicated including the 3 controls, ie. ML4, Ax6Ø and B92 each with uninoculated water agar plugs, a total of 63 petri-dishes.

The single phase technique was also used as an assay system to assess the interaction between the remaining fluorescent bacterial isolates and a single strain of A. bisporus, B92. Malt extract plus B. subtilis medium as well as standard malt extract medium were used as artificial substrates. Interactions between the fluorescent bacterial isolates, CM18, TG8, MM4, MM6 and an unknown species of Trichoderma and Fusarium were also examined. These fungal isolates were cultured on potato dextrose agar. Duplicates of each isolate were set up including controls and the plates examined every day for a week.

(2) Hume and Hayes's 2 phase method for 'strandy' strains was also used. One half of a 9 cm divided plastic petri-dish was filled with malt extract agar (2Ø ml), inoculated at 2 points 5 cm apart with strain B92 and

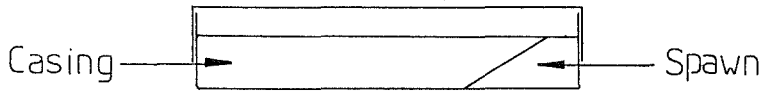
incubated at 25 C until the developing colonies reached the central partition. The second half of the petri-dish was then filled with 20 ml of water agar (1.5%) and given a further period of incubation at 25 C until half of the water agar was colonized. At this stage 2 water agar discs containing one of the 6 bacterial isolates used in (1) were placed on the water agar half of the petri-dish 6-8 mm from the advancing mycelium and the petri-dish incubated at 18 C at relative humidity between 50 and 70%. Each treatment, eg. mushroom strain B92 plus bacterial isolate CM18, was triplicated. The control which consisted of B92 plus uninoculated water agar plugs was also triplicated.

2.2.9b The method of Peerally (1979)

Peerally's modification of Eger's (1961) 'Halbschalentest' was investigated. Mushroom strains, ML4, B92, 208 and their interaction with 2 bacterial isolates, CM18 and MM4 were examined. Grain spawn was prepared and 10 g placed against one edge of a glass petri-dish (120x20 mm), casing material was applied to the rest of the dish (Fig 2.1). A turbid suspension of bacteria was prepared by suspending cells (cultured as in 2.2.3) in sterile distilled water; 1 ml of this was applied to the spawn and 5 ml to the casing material. Sterile distilled water was applied to both autoclaved and non-sterile casing soil as a control. All petri-dishes were then wrapped in plastic bags and incubated for 5 days (25 C) before reinoculating the growing margin of the mycelium with 2 ml of appropriate bacterial suspension.

The dishes were then removed from the plastic bags and placed in a refrigerated incubator for 3 weeks at 17-18 C. The door of the incubator was opened 2-3 times a day to help reduce CO_2 levels.

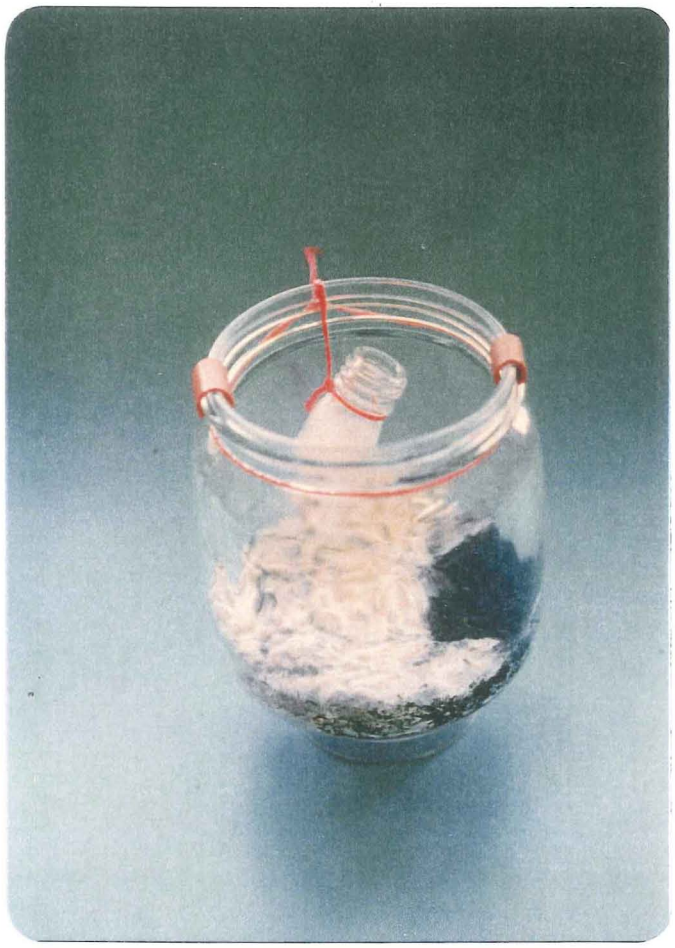
Fig 2.1. The modified 'Halbschalentest'.



2.2.9c Modification of Peerally's (1979) technique

The following modifications to the method described in section 2.2.9b were made: To ensure low levels of CO_2 'Agee' preserving jars (300 ml) with petri-dish lids raised slightly from the lip of the jars by 2 pieces of split rubber tubing were employed as 'growth chambers'. Bijou bottles containing KOH pellets were suspended inside the jars (Plate 2.1). Following an incubation period of 5 days (25 C) the jars were reinoculated as in section 2.2.9b and incubated at 15-18 C. To prevent the casing material from drying out 3 ml of sterile distilled water was applied to the material every second day. The interaction between mushroom strains B92 amd ML4 and bacterial isolates MM4 and CM18 were examined using this technique. Controls of sterile and non-sterile casing material inoculated with sterile distilled water were

Plate 2.1. The modified Peerally (1979) 'growth chamber' (a petri-dish lid rests on the rubber stops) (x Ø.5).



employed as controls. After sporophore initiation and development (approximately 3 weeks) an attempt was made to reisolate bacteria from the casing materials.

2.2.9d Application of casing soil to malt extract medium

Mushroom strains, B92 and ML4 were centrally inoculated onto malt extract plus B. subtilis medium in a standard 9 cm diameter plastic petri-dish and incubated at 25 C for 14 days. The mycelium was then covered with a layer of sterile casing soil, inoculated with 5 ml turbid bacterial suspension and incubated at 17-18 C in a refrigerated incubator. Bacterial isolates MM4 and CM18 were used and plates, including controls, duplicated.

2.2.1Ø THE EFFECT OF BACTERIA ON MYCELIAL GROWTH

Approximately 1Ø g of grain spawn (Strain B92) was placed in the bottom of a glass tube (3Ø mm diameter) stoppered with cotton wool. Sterile casing soil was applied to a depth of 5 cm and inoculated with 5 ml turbid bacterial suspension (CM18). The tubes were incubated at 25 C. Triplicate tubes were set up and measurements taken every 2-3 days.

2.2.11 SCREENING FOR MERCURY RESISTANT BACTERIA

2.2.11a Filter-paper disc assay

Petri-dishes containing 2Ø ml of LB medium were divided into 8 and Ø.Ø5 ml of a bacterial suspension (a turbid

suspension diluted 1:25) was spread over each partition and allowed to dry. Sterilized 5 mm antibiotic assay discs were moistened with 12 ul of a 1 mg/ml $HgCl_2$ solution, left 3Ø min to dry and placed in the centre of each partition. The petri-dishes were incubated at 3Ø C and examined after 24 h (Linton 1983, Potter 1985). $HgCl_2$ free discs were employed as controls.

2.2.11b Mercury containing media

Petri-dishes containing LB medium plus $HgCl_2$ (12 ug/ml) (pH 7.2) were inoculated with Ø.1 ml of a $1x1Ø^8$ cell/ml bacterial suspension. The plates were incubated at 3Ø C and examined after 24 h (Potter 1985). P. aeruginosa containing the FP2 (Hg resistant (Hg^+)) plasmid and P. aeruginosa without the FP2 plasmid ie. Hg sensitive (Hg^-) were used as controls.

2.2.12 PLASMID CURING USING MITOMYCIN C

Plasmid borne characters are usually unstable and are spontaneously lost or cured at an observable rate. This level of instability can be increased in order to prove that characters are plasmid borne. Agents which either prevent plasmid replication or cause pili to dissociate (preventing cells which have lost plasmid(s) from being reinfected), are used as curing agents (Martin 1982). Mitomycin C, an anti-tumour antibiotic complex, is normally used as a curing agent for Pseudomonas (Rheinwald et al. 1973).

The (Hg^+) isolate MM4 was cultured in LB broth

containing 1, 1Ø and 25 ug/ml mitomycin C. After 24 h on a rotary shaker (3Ø C) bacteria from the 25 ug/ml flask (near lethal concentration) were streaked onto LB plates. After a further 24 h period single colonies were transferred to both a master plate and to a LB plate containing $HgCl_2$ (12 ug/ml) in order to score and select individual clones of the Hg^- phenotype. Hg^- colonies were restreaked on LB medium and single colonies rechecked for their sensitivity to Hg by replating onto LB plus $HgCl_2$ medium. (Rheinwald et al. 1973, Tucker and Pemberton 1978).

2.2.13 EXAMINATION OF THE EFFECT OF A MITOMYCIN C TREATED (PLASMID CURED) FLUORESCENT BACTERIAL ISOLATE (MM4) ON SPOROPHORE INITIATION

The single phase system described in section 2.2.9 (1a) and also the modified method of Peerally (1979), section 2.2.9 (3) (using A. bisporus strain ML4) were used to assess the effect of Hg^+ and Hg^- phenotypes of MM4 on mycelial growth and sporophore initiation. Each treatment was triplicated including controls.

2.2.14 ISOLATION OF PLASMID DNA

2.2.14a Alkaline lysis

Based on the technique in Maniatis et al. (1982) and modified by H.K. Mahanty, Dept. Plant and Microbial Sciences, University of Canterbury, NZ.

Cells were cultured overnight in 5 ml LB broth (where

appropriate this contained $HgCl_2$ (12 µg/ml)) and 1.5 ml transferred into Eppendorf tubes and spun (3 min) in a microfuge. The supernatant was discarded and the pellet resuspended in 6Ø µl of solution I (+ lysozyme) and placed on ice for 2Ø min. Freshly prepared solution II (12Ø µl) was then added and the resulting solution mixed gently with the tip of a Gilsen pipette. After 5 min on ice 9Ø µl of solution III was added and the tubes, following mixing, stored at room temperature for a further 5 min. The tubes were then placed on dry ice till frozen, then spun for 12 min at 4 C. The supernatant (22Ø µl) was transferred to clean Eppendorf tubes, Ø.6 volumes of cold isopropanol added and these new tubes placed back on dry ice till frozen. After further microfuging (12 min, at 4 C) the supernatant was discarded, the tubes blotted on tissue paper, refilled (1.5 ml) with cold 7Ø% ethanol and spun for 2 min at room temperature. The supernatant was poured off and the tubes dried in a vacuum desiccator. The pellet was then resuspended in 5Ø µl Te buffer (pH 8) and 1 µl RNase was added before placing the tubes in a water bath at 37 C for 1Ø min. Cold ethanol was added and the tubes placed once again on dry ice till frozen, centrifuged 12 min at 4 C and dried in a vacuum desiccator. The DNA pellet was resuspended in 5Ø µl Te buffer (pH 8) and 4 µl taken for gel analysis.

A variation of this method which involed a phenol extraction step prior to alcohol precipitation was also used.

Two strains of P. aeruginosa, one with and one minus the FP2 plasmid were used as controls.

Solution I: 5ØmM glucose
25mM Tris.Cl (pH 8)
1ØmM EDTA
+ lysozyme added just before use

Solution II: Ø.2N NaOH
1% SDS
made fresh

Solution III: 6Ø ml 5M potassium acetate
11.5 ml glacial acetic acid
28.5 ml distilled water

2.2.14b Rapid disruption of colonies

Based on the method in Maniatis et al. (1982) and modified by H.K. Mahanty, Dept. Plant and Microbial Sciences, University of Canterbury, NZ.

Bacteria were grown on LB medium (where appropriate this contained $HgCl_2$ (12 µg/ml)) until single colonies were 2-3 mm in diameter. A small quantity of a single colony was scraped off with a sterile toothpick and transferred to a master plate and the remainder placed in the well of a spotting tray containing 25 µl lysis mixture. The cells were mixed gently with a toothpick untill lysis occurred then 5 µl of 6 x loading buffer was added and 12 µl of the final mixture loaded into a gel for analysis.

Lysing solution: 4.1 ml 2N NaOH
Ø.6Ø5 g Tris base
3 g SDS
made to 1ØØ ml with sterile distilled water.

2.3 RESULTS

2.3.1 ISOLATION AND IDENTIFICATION OF BACTERIAL ISOLATES

Enrichment of a number of different substrates (Table 2.2) yielded 34 fluorescent bacterial isolates. Reference cultures (Table 2.1) were obtained and examined together with these isolates. The 34 isolates plus 5 reference organisms examined possessed the following characteristics in common: All were Gram negative, non-fermentative, oxidase positive, oxidative, metabolized glucose, produced a fluorescent pigment on either KB or KA and motile by polar multitrichous flagella (except P. aeruginosa which has polar monotrichous flagellae). All were able to produce arginine dihydrolase and all grew at 4 C with the exception of P. aeruginosa. No isolate was able to hydrolyse starch or produce H_2S. With the exception of P1 and ST1 none were able to produce levan from sucrose. Colonies were smooth, cream coloured, circular and convex with the margins tending toward undulate. The margin of isolate MM6 was lobate. The organisms ranged between rod and oval/rod shape with dimensions approximately Ø.8-1.2 by 1.6-3.Ø μm. A number of characters were dissimilar and are given in Table 2.3.

On the basis of the above characteristics the 34 isolates were considered to be members of the genus Pseudomonas (Hendrie and Shewan 1979). Of these, 32 were considered to be members of the P. putida group on the basis that they failed to hydrolyse gelatin and denitrify. The

Table 2.1. Source of reference cultures.
(with reference to Table 2.3)

Isolate no.	Identification	Source	Origin
2758	P. putida (P. put)	PDD, DSIR, Auckland, NZ	Indiana, USA ATCC 12633
348Ø	P. fluorescens (P. flu)	PDD, DSIR, Auckland, NZ	Whangarei, NZ L. sativa
B56	P. tolaasii (P. tol)	University of Canterbury	Christchurch, NZ A. bisporus
B57	P. gingeri (?) (P. gin)	University of Canterbury	Christchurch, NZ A. bisporus
-	P. aeruginosa (P. aer)	Christchurch Hospital, Pathology Lab.	Christchurch, NZ

Table 2.2. Source of isolates
(with reference to Table 2.3)

Isolate	Source
CM	Casing layer, DSIR, Lincoln, NZ
P	Pond, University of Canterbury, NZ
S	Soil, Christchurch, NZ
ST	Avon River, Christchurch, NZ
SM	Mud, Christchurch, NZ
TG	Casing layer Ashlin Mushrooms, Lincoln, NZ
MM	Casing layer Meadow Mushrooms, Prebbleton, NZ

Additional key to table 2.3

$+_a$ = slight liquification after 1 week

+/- = small amount of growth

P. put_b = characteristics of P. putida from Bergy's Manual.

Table 2.3. Dissimilar characteristics of 34 fluorescent isolates plus 5 reference cultures

Isolate	Pigment (diffusible)	Nitrate reduction NO_3-NO_2	Nitrate reduction NO_3-N_2	Gelatin	Growth at 41 C
P. put	Yellow/green fluorescent on KB	-	-	-	-
P. put_b	Yellow/green fluorescent on KB	-	-	-	-
P. flu	Yellow/green fluorescent on KB	-	-	+	-
P. aer	Yellow/green fluorescent on KB Blue/green fluorescent on KA		+	+	+
P. tol	Yellow/green fluorescent on KB	-	-	+	-
P. gin	Yellow/green fluorescent on KB	-	-	$+_a$	-
CM4	Yellow/green fluorescent on KB	+	-	-	+
CM6	Yellow/green fluorescent on KB	+	-	-	+/-
CM8	Yellow/green fluorescent on KB	+	-	-	+
CM13	Yellow/green fluorescent on KB	+	-	-	-
CM18	Yellow/green fluorescent on KB Orange non-fluorescent on KB	-	-	-	+/-
CM18a	Yellow/green fluorescent on KB	-	-	-	+/-
P1	Yellow/green fluorescent on KB Orange non-fluorescent on KB	+	-	+	-

Table 2.3 continued

Isolate	Pigment (diffusible)	Nitrate reduction NO_3-NO_2	Nitrate reduction NO_3-N_2	Gelatin	Growth at 41 C
S1	Yellow/green fluorescent on KB	+	-	-	-
ST1	Yellow/green fluorescent on KB Orange non-fluorescent on KB	+	-	+	-
SM1	Yellow/green fluorescent on KB	-	-	-	-
SM2	Yellow/green fluorescent on KB	-	-	-	-
TG1	Yellow/green fluorescent on KB	+	-	-	+
TG2	Yellow/green fluorescent on KB	+	-	-	-
TG3	Yellow/green fluorescent on KB	-	-	-	-
TG4	Yellow/green fluorescent on KB	+	-	-	+/-
TG5	Yellow/green fluorescent on KB	+	-	-	-
TG6	Yellow/green fluorescent on KB	+	-	-	+/-
TG7	Yellow/green fluorescent on KB	+	-	-	-
TG8	Yellow/green fluorescent on KB	+	-	-	+
TG9	Yellow/green fluorescent on KB	+	-	-	+
TG1Ø	Yellow/green fluorescent on KB	+	-	-	+
TG11	Yellow/green fluorescent on KB	+	-	-	-

Table 2.3 continued

Isolate	Pigment (diffusible)	Nitrate reduction NO_3-NO_2	Nitrate reduction NO_3-N_2	Gelatin	Growth at 41 C
TG12	Yellow/green fluorescent on KB	+	-	-	-
MM1	Yellow/green fluorescent on KB	-	-	-	-
MM2	Yellow/green fluorescent on KB	-	-	-	-
MM3	Yellow/green fluorescent on KB	-	-	-	+
MM4	Yellow/green fluorescent on KB	-	-	-	+
MM5	Yellow/green fluorescent on KB	-	-	-	-
MM6	Yellow/green fluorescent on KB Blue/green fluorescent on KA	+	-	-	+/-
MM7	Yellow/green fluorescent on KB	+	-	-	+
MM8	Yellow/green fluorescent on KB	-	-	-	-
MM9	Yellow/green fluorescent on KB	-	-	-	+
MM1Ø	Yellow/green fluorescent on KB	-	-	-	+/-
MM11	Yellow/green fluorescent on KB	-	-	-	+

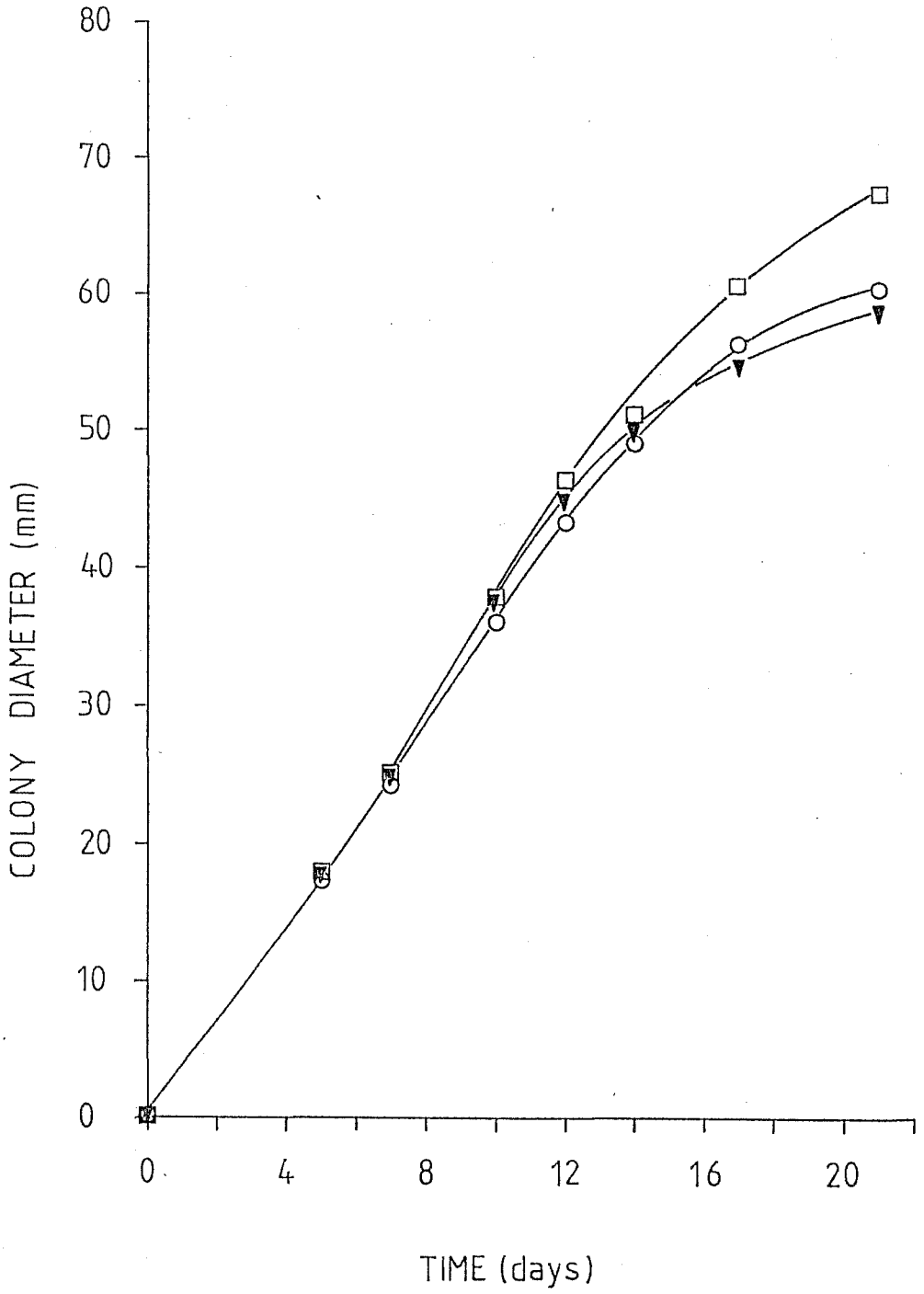
remaining 2 isolates (isolated from river and pond water, Table 2.2) were able to liquify gelatin, produce levan from sucrose and produce an orange non-fluorescent pigment on KB. They were therefore considered to belong to P. fluorescens biotype E (Doudoroff and Palleroni 1974). The strict assignment of the P. putida isolates into biotypes (A and B) requires detailed study of carbon source utilization which was not attempted, nor seen as necessary for this study, nevertheless, as all isolates grew at 4 C it is likely that the majority of them belong to biotype B (Stanier et al. 1966).

The reference cultures examined gave results consistent with their designated classification, thus, strengthening the validity of these results.

2.3.2 ASSESSMENT OF MEDIA

Analysis of variance (after 21 days incubation) revealed that the 3 media examined had a significant ($P < Ø.Ø1$) effect on the mean colony diameters of A. bisporus. Newman-Keuls test for unplanned comparisons between means showed that A. bisporus growing on malt extract plus B. subtilis medium had a significantly larger colony diameter after 21 days than A. bisporus growing on either malt extract plus P. putida or malt extract media. Fig 2.2 illustrates the mean growth rate of the colonies on the 3 different media and Plate 2.2 shows the effect of the media on growth characteristics. Malt extract plus B. subtilis medium

Fig 2.2. Growth of mushroom mycelium on 3 different media.

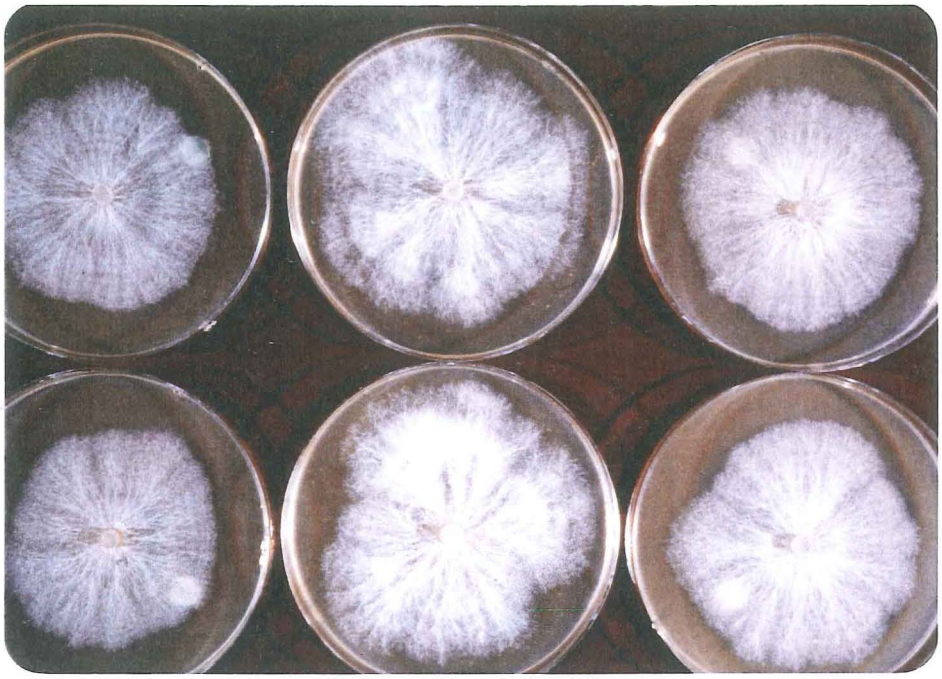


□ = B. subtilis + malt extract medium

▼ = Malt extract medium

○ = P. putida + malt extract medium

Plate 2.2. Growth characteristics of A. bisporus on 3 different media:



Malt extract	Malt extract + B. subtilis	Malt extract + P. putida

promoted vigorous strandy growth, whereas the growth on malt extract medium, especially after 14 days incubation, was very slow and wispy, a plate was seldom fully colonized before the agar began to desiccate.

2.3.3 IN VITRO FRUITING OF A. bisporus

2.3.3a The methods of Hume and Hayes (1972)

(1) The single phase method proved unsatisfactory as a means of allowing primordia production in vitro, however, many of the fluorescent isolates demonstrated their ability to inhibit the growth of A. bisporus mycelium (Plate 2.3). The results of the interactions between the fluorescent bacterial isolates and A. bisporus mycelium are presented in Table 2.4. The interaction between the two organisms was unaffected by the medium used (malt extract and malt extract plus B. subtilis). None of the fluorescent bacterial isolates which inhibited the growth of A. bisporus were able to inhibit the growth of Trichoderma or Fusarium. No primordia were formed under axenic conditions.

(2) The 2 phase technique for the production of primordia in vitro was also unsuccessful, the mycelium failing to grow past the central partition and onto the water-agar.

2.3.3b The method of Peerally (1979)

After 3 weeks incubation at 17-18 C the casing material was well colonized, however, no primordia were formed. Inoculation of the growing mycelium with the appropriate

Plate 2.3. The interaction between 6 different *P. putida* isolates and *A. bisporus* mycelium.

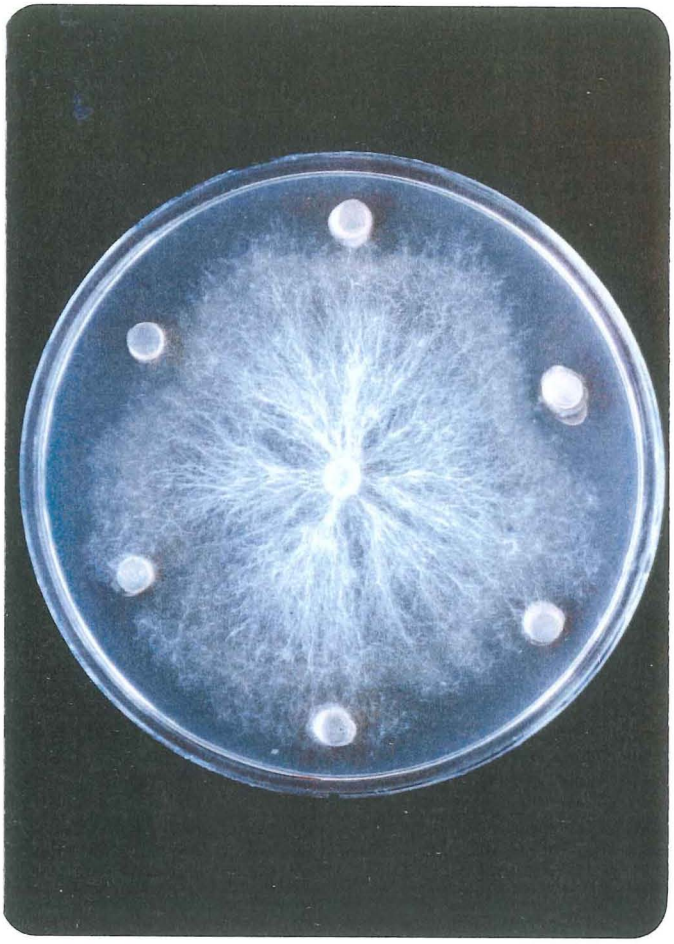


Table 2.4. Interaction between fluorescent bacterial isolates and A. bisporus mycelium (strain B92)

Fluorescent bacterial isolates	Result of interaction
TG3, TG1Ø, CM4, CM6, CM8, CM13, CM18, MM2, MM3, MM4, MM5, MM7, MM1Ø, MM11, S1, ST1, P1	+
CM18a, TG4, TG5, TG6, TG7, TG8, TG11, TG12, MM8, MM9	+/-
P. put, TG1, TG2, TG9, MM1, MM6, uninoculated discs, 2 Gram negative and 2 Gram positive (non pseudomonad) isolates from casing soil	-

+ = Clear inhibition of mycelium after 5 days (3-5mm zone)

+/- = Slight inhibition of mycelium after 5 days

- = Mycelium not inhibited

Tables 2.1, 2.2 and 2.3 give a full description of the bacterial isolates.

Table 2.5. The effect of bacteria on mycelial growth

Condition of casing material	Growth (mm) after: 4 days	7 days	1Ø days
Non-sterile	7	1Ø	18
Autoclaved plus CM18	23	4Ø	53
Autoclaved plus MM4	25	39	56
Autolaved	19	36	48

bacterial suspension did not inhibit mycelial growth. Non-sterile casing soil also failed to stimulate primordium production. This technique, nevertheless, highlighted the effect of the fluorescent bacterial isolates on mycelial growth. Under sterile conditions the mycelium was 'cotton wool' like, while under non-axenic conditions the mycelium was coarse and strandy. Sterile casing plus either MM4 or CM18 stimulated only slight strand formation.

The effect of these fluorescent bacterial isolates on mycelial growth was further emphasized by the growth of A. bisporus in tubes (2.2.1Ø) (Plate 2.4). Measurement of the growth rate revealed the stimulatory nature of both CM18 and MM4 on the rate of growth of A. bisporus mycelium (Table 2.5).

2.3.3c Modification of Peerally's (1979) technique

This technique was successful. Primodia were produced by mushroom strain ML4 1Ø-12 days after inoculation of the growing mycelial margin with either CM18 or MM4. Strain ML4 also produced a small number of primordia in non-sterile casing soil (Table 2.6). Primordia formed along the line of inoculation, and an average of 1 primordium per jar developed into a mature sporocarp capable of liberating spores (Plate 2.5). Sporophore initiation was not accompanied by a check in mycelial growth. Subsequent reinoculation of the mycelial margin failed to inhibit growth or stimulate further primordia production. A number of jars received localized inoculation of bacteria and in these cases primordium

Plate 2.4. A. bisporus growing in glass tubes through:

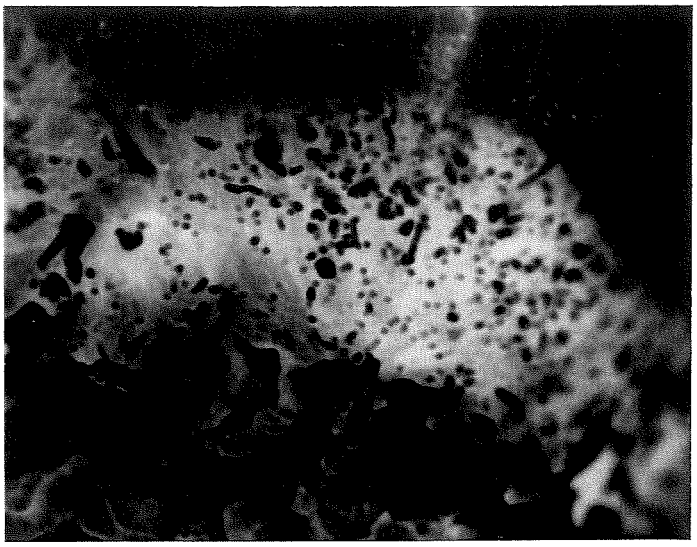
(1) Sterile casing soil.

(2) Sterile casing soil + bacterial isolate CM18

(3) Non-sterile casing soil.

Plate 2.4.

(1)



(2)

(3)

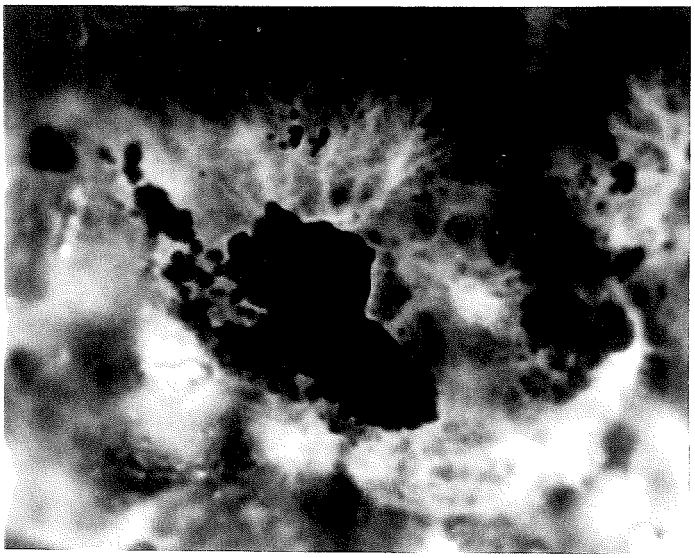


Table 2.6. The effect of 2 fluorescent bacterial isolates (CM18 and MM4) on growth rate and primordia formation in 2 strains of A. bisporus (ML4 and B92)

Treatment	Mean linear growth (mm) after 16 days	Number of primordia[1] per jar after 16 days		
		1	2	3
ML4 + CM18	63	27	35	29
ML4 + MM4	65	39	31	29
ML4 sterile	55	Ø	Ø	Ø
ML4 non-sterile	32	Ø	Ø	3
B92 + CM18	57	Ø	Ø	Ø
B92 + MM4	64	Ø	Ø	Ø
B92 sterile	58	Ø	Ø	Ø
B92 non-sterile	28	Ø	Ø	Ø

1 = Primordia were defined as smooth hyphal aggregates > 2 mm diameter.

Table 2.7. The occurrence of mercury resistance

Fluorescent bacterial isolate	Presence or absence of mercury resistance
P. aeruginosa (FP2), MM4, CM18, TG4	+
MM1, MM11, TG3, TG9, TG1Ø	+/-
P. aeruginosa, P. putida, TG1, MM2, CM4	-

\+ = resistant isolate

+/- = an inhibition zone present, but some isolated colonies growing within this zone.

\- = Sensitive isolate

Plate 2.5. Production of sporophores in a modified Peerally (1979) 'growth chamber' (x 1.5).

Plate 2.6. A. bisporus mycelium growing on sterile casing soil.

Plate 2.5.

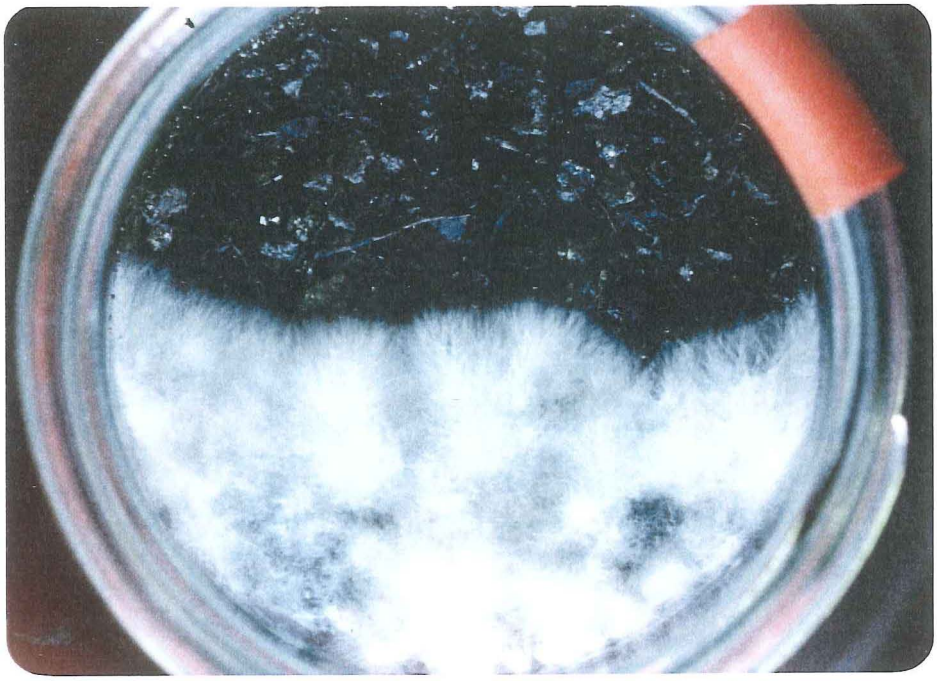


Plate 2.6.

formation was also localized. No primordia were formed by strain B92 or by either B92 or ML4 in axenic culture. The effect of bacteria on mycelial growth was as described in section 2.3.3b, refer also to Plates 2.6, 2.7 and 2.8. At the end of the experiment (after ca. 3 weeks incubation at 15-18 C) fluorescent bacteria were reisolated from the casing materials to which they had been previously added. No bacteria were isolated from the autoclaved casing material.

2.3.3d Application of casing soil to malt extract medium

The mycelium failed to grow significantly and the casing was rarely colonized.

2.3.4 MERCURY RESISTANCE

Refer to Table 2.7.

2.3.5 PLASMID CURING

Following treatment of MM4 (Hg^+) with mitomycin C, 95% of the single colonies examined were Hg^- (Plate 2.9).

2.3.6 THE EFFECT OF MM4 (Hg^+) AND MM4 (Hg^-) ON SPOROPHORE INITIATION AND MYCELIAL GROWTH IN A. bisporus

2.3.6a The single phase method of Hume and Hayes (1972)

No differences were observed between the Hg^+ and the Hg^- phenotypes of MM4 using this technique, both inhibited the growth of A. bisporus mycelium.

Plate 2.7. A. bisporus mycelium growing on sterile casing soil inoculated with P. putida isolate MM4. Note primordia (x 1.5).

Plate 2.8. A. bisporus mycelium growing on non-sterile casing soil. Note coarse strand development and sparse growth (x 1.5).

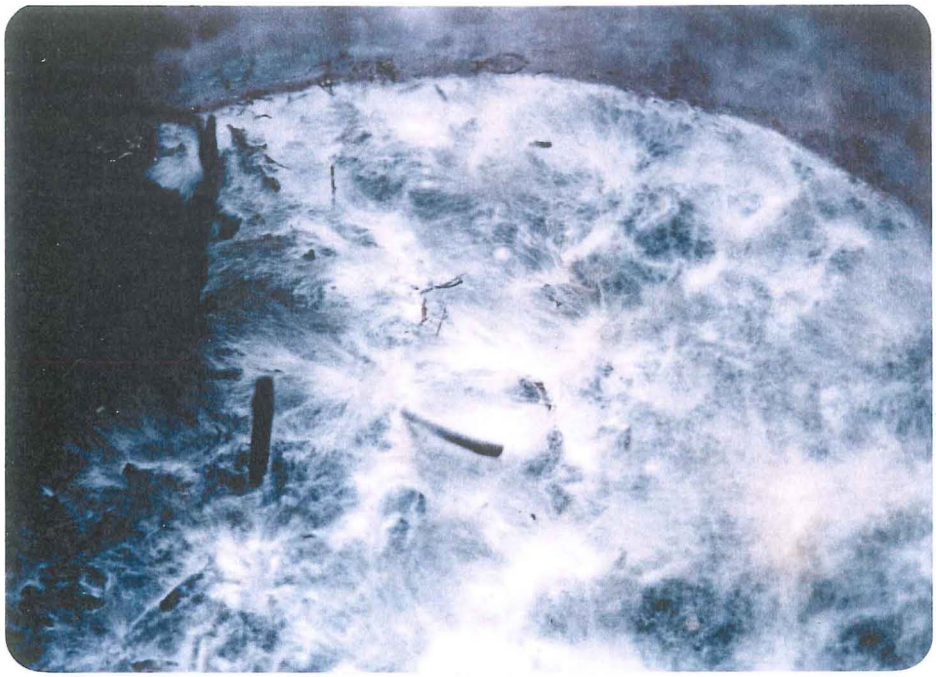
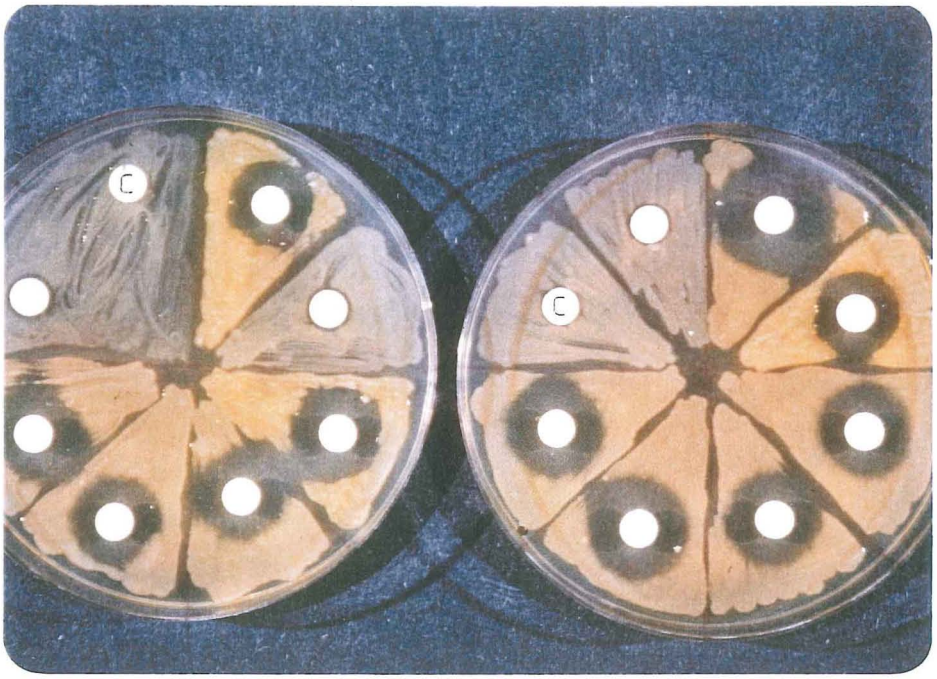


Plate 2.7.

Plate 2.8.

Plate 2.9. Results of filter paper disc assay for mercury resistance following treatment of P. putida (MM4) with mitomycin C. The bacteria (MM4) in sectors with inhibition zones have been treated with mitomycin C. Sectors which show no inhibition zones are controls (C) and non-mitomycin C treated MM4 (Hg^{+}).



2.3.6b The modified method of Peerally (1979)

Primordia were produced 10-12 days after inoculation of the growing mycelial margin with either MM4 (Hg^-) or MM4 (Hg^+), the jars treated with the latter phenotype produced a larger number of primordia (Table 2.8). The sporophore initials in the jars treated with MM4 (Hg^-) failed to grow larger than 2-3 mm in diameter and 3-4 days after initiation began to turn 'fluffy' and became over-grown with mycelium (Plate 2.10). In the jars treated with MM4 (Hg^+) many of the primordia grew to 6-8 mm diameter, several reached a diameter of 10-15 mm and 3 exceeded 20 mm, matured and liberate spores (Plate 2.11). A single, small (5 mm diameter) primordium was produced in one of the sterile controls. There was no difference between the effect of the 2 MM4 phenotypes on mycelial growth, the trends observed were similar to those reported in sections 2.3.3b and 2.3.3c (Table 2.8).

2.3.7 ISOLATION OF PLASMID DNA

2.3.7a Alkaline lysis

Plate 2.12b shows the results of a typical prepartion. P. aeruginosa without the FP2 plasmid shows a single band, while P. aeruginosa with the FP2 plasmid shows a double band. TG9, a mercury sensitive isolate shows a single band as does MM4 (Hg^-, mitomycin C treated). The 2 MM4 (Hg^+) preparations have double bands. The higher band (in those preparations which have 2 bands) is most likely plasmid DNA, while the band which has migrated slightly further and is apparent in

Table 2.8. The effect of the Hg^- and Hg^+ phenotypes of MM4 on growth rate, height of mycelium and primordia formation in A. bisporus (strain ML4)

Treatment	Mean linear growth after 16 days (mm)	Mean mycelial height after 6 days (mm)	Number of primordia[1] per jar after 16 days 1	2	3
ML4 + MM4 (Hg^-)	59	6.5	3	12	5
ML4 + MM4 (Hg^+)	62	5.5	35	23	19
ML4 sterile	54	12.Ø	Ø	1	Ø

1 = Primordia were defined as smooth hyphal aggregates > 2mm diameter.

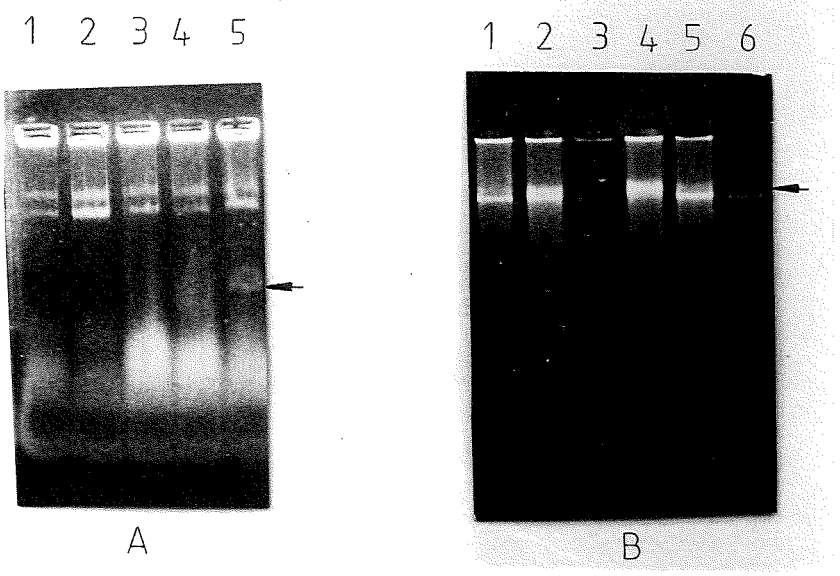
Plate 2.1Ø. A. bisporus mycelium inoculated with mitomycin C (plasmid cured) P. putida (MM4). Note overgrown primordia (x 2).

Plate 2.11. A. bisporus mycelium inoculated with P. putida (MM4) (non-mitomycin C treated) (x 1.5).

Plate 2.1Ø.

Plate 2.11.

Plate 2.12. Agarose gels showing the results of P. putida plasmid preparations.



A - Rapid disruption of colonies:

Lanes 1,2,3,4 are pseudomonads (no plasmid revealed).
Lane 5 is E. coli, plasmid (PBR322) band is visible.

B - Alkaline lysis:

Lane 1 P. aeruginosa
Lane 2 P. aeruginosa (FP2 plasmid)
Lane 3 isolate TG9 (Hg^+)
Lane 4 isolate MM4 (Hg^+)
Lane 5 isolate MM4 (Hg^-)
Lane 6 isolate MM4 (Hg^-)

←— Shows plasmid (?) band.

all lanes, can only be cellular debris and maybe a small amount of chromosomal DNA, although the extraction procedure is such, that the presence of chromosomal DNA is very unlikely (pers. comm. H.K. Mahanty, Dept. Plant and Microbial Sciences, University of Canterbury, Christchurch.).

The existence of large plasmids in pseudomonads makes the extraction and purification of plasmid DNA difficult. Problems encountered, particularily during the lysis stage of extraction, meant that the extracted DNA was not as 'clean' as it should have been. Consequently the bands on the gels were often blurred, making it difficult to predict the extact nature of the bands. Nevertheless, the presence of double bands in preparations that were expected or (known to) to contain plasmids provides good evidence for their existence. The 'would be' plasmid bands migrated only a small distance from the wells, indicating the existence of large plasmid(s), see Panopoulous et al. (1979) and Hansen and Olsen (1978). Further preparation of plasmid DNA using techniques specifically for Pseudomonas is required in order to provide more evidence for the existence of plasmids in these isolates.

2.3.7b Rapid disruption of colonies

This technique was unsuccessful for the detection of plasmid DNA in the Pseudomonas species examined. It did, however, reveal an E. coli (PBR322) plasmid (Plate 2.12a).

2.4 DISCUSSION

2.4.1 BACTERIAL ISOLATES

The ability of a large number of the P. putida isolates to grow at 41 C contrasts with the description of the species given by Stanier et al. (1966) and Doudoroff and Palleroni (1974) who use this criterion as one means of distinguishing P. aeruginosa from the P. putida-fluorescens group. The fact, however, that these isolates were able to grow at 4 C, did not produce a pigment on KA, hydrolyse gelatin or denitrify separates them convincingly from P. aeruginosa. Stanek (1972) and Fermor et al. (1979) reported the presence of Pseudomonas sp. in mushroom composts that were able to grow at 51 C. It is possible that the high temperatures generated during composting may induce mutations or select strains of P. putida able to survive at elevated temperatures.

Approximately 50% of the P. putida isolates examined were able to produce nitrite from nitrate, a characteristic which is compatible with the P. putida group (Doudoroff and Palleroni 1974).

Only 2 isolates, CM18 and MM6, failed to clearly fit into either the P. putida or the P. fluorescens group. CM18 produced an uncharacteristic orange pigment on KB and MM6 was able to produce a pigment on KA, as both lacked the ability to hydrolyse gelatin they were considered to be more putida-like than fluorescens-like. A study of the carbon sources used for growth may reveal more information regarding their

classification.

2.4.2 ARTIFICIAL MEDIA FOR THE CULTURE OF A. bisporus

The addition of autoclaved B. subtilis cells to malt extract medium proved a successful way of increasing the rate of growth and vigour of A. bisporus mycelium on an artificial substrate. It appears that A. bisporus was unable to gain any additional nutritive value from autoclaved P. putida.

It is possible in this situation where the bacteria were not the sole carbon and nitrogen sources in the media that the growth stimulus was a result of nutrients or growth factors adhering to the surface of B. subtilis, rather than degradation of insoluble polymers as has been reported by Fermor and Wood (1981). Further investigation into the involvement of bacteria in the nutrition of fungi is required to hopefully provide media better suited to the artificial cultivation of the higher fungi.

2.4.3 IN VITRO FRUITING OF A. bisporus

Hume and Hayes (1972) showed that primordia formation on artificial media (as in their in vitro techniques) was usually accompanied by the inhibition of mycelial growth. Inhibition of mycelial growth was noted in this study which suggested that the bacterial isolates were not responsible for the failure of these methods to encourage production of primordia in vitro. Wood (1976) and Eger (1972) demonstrated marked differences in the ability of mushroom strains to

produce primordia *in vitro*. It is quite probable that the conditions provided by the methods of Hume and Hayes, especially with repect to CO_2 concentrations, while favouring primordia production in the strains they used, did not meet the exacting requirements of the mushroom strains employed in this study.

Inhibition of mycelial growth was dependent on the survival of the bacterial isolates. A problem with the techniques of Hume and Hayes arose from the inability of the *P. putida* isolates to survive for more than 4-7 days on malt extract media. Consequently the isolates placed in the (+/-) category of Table 2.4 probably have the potential to inhibit mycelial growth to the same extent as those in category (+), but the early death of these isolates when placed on the malt extract media meant they demonstrated only slight inhibition.

The fact that not all of the *P. putida* isolates were able to inhibit mycelial growth tends to indicate that the factor(s) responsible for triggering sporophore initiation may be plasmid borne. Considering that plasmids are easily lost (Martin 1982), it is possible that the isolates unable to inhibit mycelial growth are without the 'sporophore inducing plasmid(s)'. In this context it is interesting to note that the reference strain of *P. putida* (known to be devoid of plasmids) did not inhibit mycelial growth, nor did isolate MM6 which was able to produce a fluorescent pigment on KA.

Attention to CO_2 levels and the creation of 'in vivo-like' conditions appear to be the likely reasons for the success of the modified Peerally method in providing conditions favourable for sporophore initiation. High CO_2 levels within the 'growth chambers' in the method of Peerally (1979) and in the 'cased malt extract media' method were possibly responsible for their failure. The fact that strain B92 would not produce primordia in the modified Peerally method further highlights the differences in the ability of mushroom strains to produce primordia in vitro.

The results obtained through the use of the modified Peerally method confirm the original work of Eger (1961), that bacteria in the casing material are indespensible in the fruiting of A. bisporus. The claim of Hayes et al. (1969) that the stimulatory bacteria in the casing material are, or are closely related to, P. putida was also confirmed.

The effect of P. putida isolates MM4 and CM18 on mycelial growth varied depending on the media upon which they were cultured. On agar plates, ie. the single phase system of Hume and Hayes (1972), MM4 and CM18 inhibited mycelial growth, yet the opposite occurred when they were applied to autoclaved casing material. Elliott and Lynch (1985), using an in vitro bioassay (similar to that of Hume and Hayes (1972)) in order to examine the effect of pseudomonads on wheat root elongation found that such a technique was not reliable and did not provide a true indication of the in vivo situation. Wong and Baker (1984) encountered similar problems when using an agar plate technique to examine the

interaction between fluorescent pseudomonads and two fungal pathogens of wheat. Considering the stresses on the bacteria and the fungus in Hume and Hayes's extremely artificial environment and the versatility of pseudomonads (Stanier et al. 1966) then it is quite possible that the mechanism operating in this system is different to that which normally operates in a mushroom house or the 'in vivo-like' conditions of the modified Peerally system.

The stimulation of both mycelial growth and sporophore initiation by P. putida confirms the results of Eger (1972) and Urayama (1967). The results of Eger (1961) and Peerally (1979) appear to conflict with the findings of this study and the above workers, however, closer investigation reveals that this is not the case. Both Eger (1961) and Peerally (1979) observed coarse strand development and mycelial inhibition followed by sporophore initiation after inoculation of pure cultures of A. bisporus with mixed bacterial populations. Similar observations were made in this study when A. bisporus was grown on non-sterile casing material. Neither Eger (1961) nor Peerally (1979) applied single isolates to sterile casing material as was done by Eger (1972), Urayama (1967) and in this study. This strongly suggests that coarse strand development and inhibition of mycelial growth is caused by bacterial species within the casing layer other than P. putida. Mycelial strands are known to form profusely in non-sterile soil (Mathew 1961) and may serve as a means of protection against other organisms including anti-mycotic-

producing bacteria (Thompson 1984) In contrast to Peerally (1979), it may be that strand development in non-sterile casing soil is a response by A. bisporus to bacteria (other than P. putida) in the casing layer and has little to do with sporophore initiation. Peerally's (1979) results also suggest that many organisms in the casing layer microflora play no part in sporophore initiation. Peerally found greater initiation of primordia when a pure culture of A. bisporus was inoculated with a mixed bacterial population selected by a bicarbonate media, than when the culture was inoculated with a mixed bacterial population selected by an acetone based media. Nair et al. (1976) and Hayes and Nair (1976) demonstrated that P. putida is favoured by a bicarbonate media, indicating that in Peerally's case, the increased primordia promoting ability of the bicrbonate population was presumably because of increased numbers of P. putida in this population. The 'inhibitory bacteria' may play a beneficial role after sporophore initiation by preventing mycelial overlay of the casing layer, but a detrimental role prior to fruiting. Thus, the addition of a suspension of P. putida to a sterile casing layer (in order to stimulate mycelial growth and trigger sporophore initiation) followed by a suspension of inhibitory bacteria after fruit body initiation (to prevent mycelial overlay) may provide, subject to further investigation, a means of reducing the length of time to the first flush and allow greater control over cropping.

The results of Peerally (1979) which led him to postulate the non-involvement of volatiles (other than CO_2)

in sporophore initiation was confirmed by this study. The conclusions reached, however, by Eger (1961), Peerally (1979) and others who placed emphasis on the abiltiy of activated charcoal to replace the effects of bacteria in fruiting, are more uncertain. Activated charcoal can both bind and release compounds and the exact nature of the effect remains unknown.

The single primordium produced under axenic conditions was not unexpected. Several other workers including Eger (1972), Wood (1976) and Peerally (1979) have reported this phenomenon, however, it is rare and appears to occur in only a few strains.

2.4.4 THE ROLE OF *P. putida* IN SPOROPHORE INITIATION

2.4.4a The role of plasmid(s)

Good evidence for the existence of one or more plasmids in *P. putida* was obtained. The plasmid(s) appear to carry genes which are in some way responsible for sporophore initiation and/or development in *A. bisporus*.

The fact that mitomycin C treated (plasmid cured) MM4 failed to encourage sporophore development in *A. bisporus*, while non-mitomycin C treated MM4 stimulated both sporophore initiation and development when inoculated onto pure cultures of *A. bisporus* mycelium, suggests that either (1) the plasmid borne genes play a more significant role in sporocarp development than in sporophore initiation and that the genes encoding for the factors responsible for triggering sporophore initiation are chromosomally borne, or (2) the

loss of plasmid(s) resulted in the loss of fitness of the bacterium (Levin and Lenski 1983) and consequently sporophore initiation and development was affected.

(1) Mercury resistance. The loss of mercury resistance following treatment of MM4 with an active curing agent for pseudomonads, mitomycin C, provides further strong evidence for the existence of a plasmid (Chahrabarty 1976, Martin 1982). Most significantly, the genes encoding for mercury resistance serve as a marker allowing the detection of the plasmid(s) which appears to be involved in the process of sporophore initiation and or development in A. bisporus and will prove particularily important in future work.

2.4.4b The mechanism of sporophore initiation

Little or no direct evidence exists for any of the theories which attempt to explain the mechanism by which bacteria control sporophore initiation. The 2 main theories; (1) that bacteria produce a stimulatory compound (Hayes 1972, Urayama 1967) and (2) that bacteria control fruiting by removing a fruiting inhibitor (Eger 1961 and in part Peerally 1979), appear equally valid. A number of new ideas arise from recent literature: Harwood et al. (1984) investigated chemotaxis in P. putida and found that aromatic acids serve as chemoattractants for this organism. Secondary metabolism in agarics has not been widely investigated, but evidence from the examination of secondary metabolism in similar organisms indicates that the production of aromatic acids (particularily those derived from shikimate) by A. bisporus

mycelium is almost certain (Turner 1971). It is possible, considering the nature of these compounds, that one or more of them may function as a fruiting inhibitor and that P. putida may control sporophore initiation by degrading and removing these compounds, thus, stimulating sporophore initiation provided that environmental conditions, especially CO_2 levels are favourable. It is equally possible that P. putida may produce a compound or compounds (of a non-volatile nature) which are able to stimulate sporophore initiation, the chemical attractants produced by the mycelium ensuring that the bacterium exists in close proximity to the mycelium. The ability of P. putida to degrade and utilize many different and diverse compounds (Stanier et al. 1966) adds weight to the fruiting inhibitor theory of Eger (1961). The fruiting inhibitor theory also provides a means of explaining the occasional development of primordia under axenic conditions. It is possible that in some instances an external agent, possibly air or water, may be able to remove a fruiting inhibitor, thereby initiating primordium production, (it is difficult to envisage production of a stimulatory compound by an abiotic external agent). The fact that Peerally (1979) found a greater number of primordia produced under axenic conditions in aseptically aerated flasks, than in petri-dishes where diffusion was the only means of gas exchange, indicates this could be the case.

The apparent invovement of plasmid genes in the mechanism of sporophore initiation provides a novel approach

to this complex problem. Application of the techniques of molecular biology; digestion, plasmid transfer, gene cloning and characterization etc., may aid in elucidating the mechanism underlying sporophore initiation.

2.4.4c The evolutionary significance

The evolutionary significance of the relationship between A. bisporus and P. putida has not received any attention, despite the extraordinary nature of the relationship where A. bisporus has apparently come to rely on a bacterium in order to reproduce.

A. bisporus is known to produce volatile compounds which both favour and can be used as carbon sources by P. putida (Hayes and Nair 1976). Aromatic acids, as discussed previously, appear to play a similar role, also attracting the bacterium to the mycelium. Thus, the bacterium is provided with a source of food. Presumably the mushroom also derives some benefit from the association and the likelyhood of encountering P. putida in most niches is high. P. putida is known to be widespread (Stanier et al. 1966), existing saprophytically in almost every soil and water environment. It is possible that P. putida protects the mushroom from pathogenic bacteria, especially from the closely related pathogenic pseudomonads; P. tolassii (bacterial blotch), P. agarici (drippy gill) and Pseudomonas sp. (Mummy diease), through either the possession of a bacteriocinogenic plasmid, (similar to the mechanism by which Agrobacterium rhizogenes kills A. tumefaciens (Kerr and

Brisbane 1983)) or through the transfer of a plasmid which possibly robs the pathogenic species of its virulence. It is possible that the main chromosome could be involved, especially if the relationship has evolved over a long period of time, however, highly specialized mechanisms of this type are usually plasmid borne (Timmis and Puhler 1979).

A greater understanding of the evolutionary significance of the relationship between A. bisporus and P. putida would no doubt be extremely valuable as an aid to any research aimed at elucidating the mechanism of sporophore initiation.

2.5 CONCLUSION

A number of fluorescent bacteria isolated from casing materials, soil and water were identified. The 32 isolates taken from casing materials and soil were considered to be *P. putida*, while those isolated from water appeared to belong the *P. fluorescens* group, biotype E.

A novel medium involving the addition of autoclaved *B. subtilis* cells to malt extract agar was developed. The ability of this medium to encourage vigorous growth of mushroom mycelium suggests there are benefits to be gained from investigations into the role of bacteria in the nutrition of higher fungi.

In vitro production of primordia for one strain (ML4) was achieved by modifying Peerally's (1979) method. The failure of the remaining techniques to allow *in vitro* production of primordia was probably due to the inability of these methods to provide the specific environmental conditions required by each mushroom strain. The single phase method of Hume and Hayes (1972) provided a system whereby the interaction between *A. bisporus* and *P. putida* could be examined, however, this system was extremely artificial and almost certainly did not give an indication of the *in vivo* situation.

P. putida isolates, when inoculated along-side pure cultures of *A. bisporus* growing in '*in vivo*-like' systems, were able to promote both sporophore initiation and mycelial growth.

The casing layer contains a wide range of bacterial species and the role of most of these in the process of sporophore initiation is unknown. This investigation indicates that bacteria in the casing layer, other than P. putida, are responsible for intensive strand formation and mycelial inhibition. It is possible that these bacteria are not directly involved in sporophore initiation.

Good evidence exists for the presence of one or more plasmids in P. putida isolate MM4. The plasmid(s) appears to be involved in the process of sporophore initiation and development and possesses genes which encode for mercury resistance. Further research is required in order to determine the precise nature of this plasmid(s) and its involvement in fruit body formation in A. bisporus.

CONCLUDING COMMENT

The results from this investigation further emphasize the complexity of the various interacting factors which constitute the casing layer. A number of approaches to the improvement of existing casing materials are suggested. Physical and chemical properties, to a large extent, are responsible for creating the environment in which the mushroom and microflora must coexist. Manipulation of these properties provides one way of establishing conditions within the casing layer which favour the bacteria necessary for sporophore initiation and therefore the mushroom.

Direct manipulation of the microbial flora of the casing layer offers many interesting and exciting possibilities, however, a greater understanding of the role, both singly and in combination, of the various organisms present in the casing layer is first required. The closed ecosystem based on Peerally's (1979) modification of the 'Halbschalentest' provides a sound basis for this work.

Further investigations into the role of the casing layer and especially the role of the microflora, in sporophore initiation should aim to examine the problem from different angles. This study has demonstrated the value of utilizing the tools of molecular biology to investigate the genetics of P. putida. Investigations aimed at elucidating the evolutionary significance of the symbiosis between P. putida and A. bisporus may also prove beneficial.

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APPENDIX 1

Formulae for the calculation of moisture properties

(1) Moisture factor = $\frac{\text{wt air-dry soil (g)}}{\text{wt oven-dry soil (g)}}$

(2) Gravimetric water content = $\frac{\text{wt air-dry soil (g)} - \text{wt oven-dry soil (g)}}{\text{wt oven-dry soil (g)}}$

(3) % Dry matter in air-dry soil = $\frac{\text{wt oven dry soil (g)} \times 100\%}{\text{wt air-dry soil (g)}}$

APPENDIX 1 a

Casing moisture.

CASING	Without lime			With lime		
	M.F.	G.W.C.	%D.M.	M.F.	G.W.C.	%D.M.
Pu	1.060	5.6%	94.4	1.045	4.5%	95.7
PuFm	1.096	9.6%	91.2	1.053	5.3%	95.0
Ba	1.144	14.4%	87.4	1.074	7.4%	93.1
GBS	1.130	13.0%	88.5	1.077	7.7%	92.9
Fm	1.161	16.1%	86.1	1.064	6.5%	93.9
FmBa	1.152	15.2%	86.8	1.069	6.9%	93.6
FmPe	1.163	16.3%	86.0	1.068	6.9%	93.6
Pe	1.143	14.2%	87.5	1.073	7.3%	93.2
PeBa	1.151	15.1%	86.9	1.067	6.7%	93.7

M.F. = Moisture factor

G.W.C. = Gravimetric water content

%D.M. = Percentage dry matter

APPENDIX 2

Water holding capacity (% by weight)

CASING	- lime	+ lime
Pu	72%	75%
PuFm	117%	123%
Ba	161%	125%
GBS	146%	127%
Fm	244%	145%
FmBa	187%	143%
FmPe	228%	154%
Pe	244%	163%
PeBa	236%	133%

APPENDIX 3 - MEDIA

TSI
(Anon. 1958)

Beef extract	3.Ø g
Yeast extract	3.Ø g
Peptone	2Ø.Ø g
Glucose	1.Ø g
Lactose	1Ø.Ø g
Sucrose	1Ø.Ø g
$FeSO_4.7H_2O$	Ø.2 g
$NaCl^4$	5.Ø g
$Na_2S_2O_3.5H_2O$	O.3 g
$Agar^2$	2Ø.Ø g
Phenol red, Ø.2% solution	12.Ø ml
Distilled water	1ØØØ ml
pH 7.2	

O - F medium
(Hugh and Leifson 1953)

Peptone	2.Ø g
NaCl	5.Ø g
K_2HPO_4	1.5 g
Bromothymol blue	Ø.Ø3 g
Agar	3.Ø g
Distilled water	1ØØØ ml
pH 7.2	

King's medium A
(King et al. 1954)

Peptone	2Ø.Ø g
Glycerol	1Ø.Ø g
K_2SO_4	1Ø.Ø g
$MgCl^4_2$	1.4 g
$Agar^2$	15.Ø g
Distilled water	1ØØØ ml
pH 7.2	

King's medium B
(King et al. 1954)

Peptone	2Ø.Ø g
Glycerol	1Ø.Ø g
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
$Agar^4$	15.Ø g
Distilled water	1ØØØ ml
pH 7.2	

Nitrate reduction medium
(Zarkower et al. 1984)

KNO_3	5.Ø g
Yeast extract	1.Ø g
K_2HPO_4	2.Ø g
$MgSO_4$	Ø.1 g
$Agar^4$	3.Ø g
Distilled water	1ØØØ ml
pH 7.2	

Reagent A = 8 g sulphanilic acid in 1ØØØ ml 5N acetic acid
Reagent B = 5 g naphthylamine in 1ØØØ ml 5N acetic acid

LB (Luria-Bertani) medium
(from Dillon et al. 1985)

Tryptone	1Ø.Ø g
Yeast extract	5.Ø g
NaCl	1Ø.Ø g
Agar	15.Ø g
Distilled water	1ØØØ ml
pH 7.5	

Succinate salts medium
(Krieg 1981)

Sodium succinate	4.Ø g
KNO_3	Ø.5 g
K_2HPO_4	Ø.5 g
$MgSO_4.7H_2O$	Ø.2 g
$CaCl_2.2H_2O$	Ø.1 g
$FeSO_4.7H_2O$	Ø.2 g
Agar	15.Ø g
Distilled water	1ØØØ ml
pH 7.2	

Arginine medium
(Thornley 196Ø)

Peptone	1.Ø g
NaCl	5.Ø g
K_2HPO_4	Ø.3 g
L-Arginine.HCl	1Ø.Ø g
Phenol red	Ø.Ø1 g
Agar	3.Ø g
Distilled water	1ØØØ ml
pH 7.2	